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Erythropoietin Receptor protects Lung Cancer Cells from Chemo- and Radiotherapy independent of Erythropoietin

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1. Summary

Erythropoietin (EPO) is used to treat anemia of cancer but is suspected to increase cancer mortality. EPO is also expressed in hypoxic regions of the tumor and might, thus, contribute to hypoxia-induced malignancies, including resistance to chemo- and radiotherapy. However, it is controversially discussed whether a functional EPO receptor (EPOR) is expressed by cancer cells. Thus, we analyzed the expression and function of EPOR in hypoxia-induced resistance towards chemo- and radiotherapy in lung cancer, the most common type of cancer accounting for the majority of cancer related deaths. We generated A549 and H661 EPOR knockout lung cancer cells by CRISPR/Cas and treated them with chemo- and radiotherapy under normoxic and hypoxic conditions. Our data suggests that EPOR expression is required for hypoxia-induced resistance to chemo- and radiotherapy. However, our data show that EPO is not required for EPOR-dependent cancer cell protection. We can further show that cancer cells release a protective factor that uses EPOR to protect cancer cells in a paracrine way under hypoxic conditions. This factor is included in exosomes and by using proteomics, we identified three potential candidates that might act as alternative EPOR ligands. In conclusion, our data provides the novel finding, that EPOR, either as a homodimeric or, more likely, as a heterodimeric receptor protects cancer cells from chemo- and radiotherapy with an alternative ligand.

2. Zusammenfassung

Erythropoietin (EPO) wird zur Behandlung von Krebsanämie angewendet, steht jedoch im Verdacht, die Krebssterblichkeit zu erhöhen. EPO wird auch in hypoxischen Regionen des Tumors exprimiert und kann daher zu Resistenz gegen Chemo- und Strahlentherapie beitragen. Es wird kontrovers diskutiert, ob ein funktioneller EPO-Rezeptor (EPOR) von Krebszellen exprimiert wird. Daher analysierten wir die Expression und Funktion von EPOR bei hypoxie-induzierter Resistenz gegen Therapie bei Lungenkrebs, der häufigsten Krebsart. Wir haben mit CRISPR/ Cas A549- und H661-EPOR-Knockout-Lungenkrebszellen erzeugt und diese unter normoxischen und hypoxischen Bedingungen mit Chemo- und Strahlentherapie behandelt. Unsere Daten legen nahe, dass die EPOR-Expression für eine hypoxie-induzierte Resistenz gegen Chemo- und Strahlentherapie erforderlich ist. Sie zeigen jedoch auch, dass EPO für Schutz von Krebszellen nicht erforderlich ist. Wir können weiterhin zeigen, dass Krebszellen einen Schutzfaktor freisetzen, der EPOR verwendet, um Krebszellen unter hypoxischen Bedingungen auf parakrine Weise zu schützen. Dieser Faktor ist teilweise in Exosomen enthalten. Mithilfe der Proteomik haben wir drei potenzielle Kandidaten identifiziert, die als alternative EPOR-Liganden fungieren könnten. Zusammenfassend kommen wir zum neuartigen Befund, dass EPOR entweder als homodimerer oder wahrscheinlicher als heterodimerer Rezeptor Krebszellen vor Chemo- und Strahlentherapie mit einem alternativen Liganden schützt.

3. Introduction

Erythropoietin (EPO) is a glycoprotein- hormone, which controls the production of erythrocytes from precursor cells in the bone marrow. It is a polypeptide of 165 amino acids and has a molecular mass of 30.4kDa [1]. In adults, 85-90% of the EPO is synthesized in the interstitial cells in the peritubular capillary bed of the kidneys [1]. EPO is a receptor ligand and essential for survival, proliferation and differentiation of the erythrocytic progenitors [2]. The EPO Receptor (EPOR) can exist in at least two different conformations: a homodimer, which consists of two identical EPOR subunits; or a heterodimer, comprised of an EPOR and a beta-common (βc) subunit (CD131)[3]. In red blood cells, EPO stimulates erythroid cell differentiation and survival via the EPOR homodimer [3]. So far, EPO is the only known ligand for EPOR with a high binding affinity to the homodimeric isoform of EPOR (~100–200 pmol/L [3]). The binding affinity of EPO to the EPOR heterodimer, however, is a thousand-fold lower [4-5], which suggest that other ligands might bind better to this form.

EPOR is expressed in many organs such as the brain, cardiovascular system, etc., indicating that EPO has a biological role beyond red blood cell production. The role of EPO and the heterodimeric EPOR in non-hematopoietic tissues is not fully understood but may involve regulation of proliferation and survival in non-hematopoietic tissue [3]. For example, EPO treatment reduces myocardial infarct size, protects against ischemia-reperfusion injury and promotes ventricular remodeling [2].

Therapeutically, recombinant human erythropoietin (rhEPO) is commonly used in anemic patients such as in chronic kidney disease patients to compensate the insufficient production of EPO in the kidney [6]. In cancer patients, EPO is used to treat cancer-associated or therapy-associated anemia [7], [8]. Because EPO regulates proliferation and survival, potentially also in cancer cells, application of EPO in cancer patients has been controversially discussed for years. Studies analyzing the impact of rhEPO treatment on *in vitro* cell proliferation and survival as well as the impact of EPO administration on mortality in cancer patients seem to be contradictory. While several clinical studies [9], [10], [11] do not find evidence for increased tumor progression during EPO administration, other studies show that EPO decreased survival of cancer patients [7], [12], [13]. Also, *in vitro* studies in hormone sensitive prostate cancer cells reported an increased proliferation of tumor cells in response to

EPO treatment [12], [14] and EPO gene expression has been detected in several cancer cell lines.

In vivo, EPO has been suggested to promote cancer in two ways: 1. EPO has been reported to increase tumor angiogenesis and might be required to maintain the integrity of the endothelium in tumors [2], [11], [12]. 2. EPO directly signals on cancer cells and selects cells with diminished apoptotic potential [12], [13], induces cellular proliferation [12], or protects from chemoradiation [12]. To signal on cancer cells, EPO requires the expression of EPOR, although a recent study suggests that EPO can also promote tumor growth via the ephrin-type B receptor 4 (EphB4) [15]. This is particularly interesting because the expression of EPOR in cancer cells has been doubted for many years. Although some studies reported on EPOR gene expression in cancer cell lines [118], no evidence for EPOR gene expression in tumor tissues has been reported [2]. The expression of EPOR in tumor tissues was also doubted because no antibodies against EPOR existed that reliably detect EPOR. However, recently developed antibodies against EPOR show that EPOR is expressed in both, human cancer cell lines and tumor tissue of human patients [16]. This finding fueled the ongoing debate about the role and function of EPOR in tumors.

A strong indicator of a functional EPOR in cancer cells is the inducible gene expression of both EPO and EPOR in hypoxia [2] [12]. Tumor hypoxia frequently occurs in solid tumors when they outgrow the capillary diffusion capacity resulting in tumor regions that are inadequately supplied by oxygen. Tumor hypoxia is a frequent feature of malignancies and is often associated with treatment resistance, an aggressive clinical phenotype, and a poor prognosis [12]. Hypoxia in cancer leads to a change in the microenvironment of the tumor, which promotes apoptosis and autophagy, or inhibits DNA damage and mitochondrial activity through various signaling pathways [17]. Thereby, tumor hypoxia renders tumors insensitive to immunotherapy, chemotherapy or radiation [17]. Especially the efficacy of radiation is drastically reduced by tumor hypoxia because tumor hypoxia activates DNA damage signaling as well as DNA repair pathways [125]. In addition, radiation requires oxygen to generate reactive oxygen species that damage tumor cells [126]. Further, hypoxia promotes a decrease in tumor pH, which leads to multidrug resistance through various mechanisms [17]. These include a decreased concentration of the drug due to “ion trapping,” genetic alterations (including p53 mutations),

reduced apoptotic potential, and an increased activity of the multidrug transporter p-Glycoprotein which is in charge of pumping out cytotoxins [17].

Tumor hypoxia frequently occurs in lung cancer, the leading cause of cancer related deaths in male patients, and the second among female patients worldwide [67- 69], and reduces the success of chemo- or radiation in human patients [18]. Because lung cancer patients often develop anemia [67], they are commonly treated with EPO. However, it has also been reported that co-expression of EPO/EPOR in lung cancer biopsies is a negative prognostic factor for early stage non-small lung cancer [12]. *In vitro* and *in vivo* preclinical studies show that EPOR is expressed in (A549) lung cancer cells [11], [16], but seems to be non-responsive to EPO [19]. Thus, it is possible that lung cancer cells either express non-functional EPOR or that EPOR is activated by different means.

We hypothesize that EPOR, despite its non-responsiveness to EPO, has a biological function in lung cancer cells. To test our hypothesis, we generated A549 and H661 EPOR knockout cells by CRISPR/Cas and analyzed their survival after radiation and chemotherapy by clonogenic assays under normoxic and hypoxic conditions.

4. Material and Methods

4.1 Cellculture

We cultured adenocarcinomic human alveolar basal epithelial cell lines A549 and H661 in 10 cm petri dishes. H661 were maintained in Roswell Park Memorial Institute 1640 (RPMI-1640) (Gibco by life Technologies) and A549 cells were maintained in Dulbecco's Minimum Essential medium (MEM) with Earle's Salts (Gibco by Life Technologies). Both media were supplemented with 10% Fetal Calf Serum (FCS) (heat inactivated), 2ml L-glutamine, 20mM HEPES buffer solution (Gibco by Life Technologies), 10mM Sodium Pyruvate solution (Gibco by Life Technologies) and 2ml Penicillin/ Streptomycin. The cells were split when reaching 80-100% of confluency in a 1/10 ratio. To do so, both cell lines were washed with Phosphate buffered saline (PBS) and incubated in 0.05% Trypsin (Gibco by life Technologies) for 1-2 min. The detached cells were re-suspended in 9ml of fresh 10% FCS medium. Cells were kept at 37° C in normoxia (21% O_2 and 5% CO_2) Revco (Thermo Scientific) or at hypoxia at 37°C (1% O_2 and 5% CO_2) in Hera cell 240 incubator (Heraeus).

Cell freezing

To harvest cells for freezing, they were trypsinized as described above. To remove trypsin, cells were re-suspended in 9ml of fresh 10% FCS medium in a 50ml Falcon tube. The suspension was centrifuged at 180 x g for 5 min and the supernatant was removed. The cell pellet was resuspended in a mixture of 90% FCS and 10% DMSO. This mixture was then aliquoted into Cryo vials and cells were kept in a freezing container holding isopropanol. This container was placed in a -80°C freezer to cool the cells down with 1°C per minute until -80°C were reached. Lastly, the cells were then stored in liquid nitrogen.

Cell thawing

The cells were quickly thawed by placing them into a pre-warmed water bath at 37°C. The cell suspension was then added into 9ml of fresh 10% FCS medium. To remove DMSO of the freezing mixture (90%FCS and 10% DMSO), the cells were centrifuged

at 180 x g for 5 minutes and the supernatant was aspirated. 9ml of fresh 10% FCS medium were added to the cell pellet and the suspension was transferred onto a fresh 10 cm petri dish. Cells were incubated at 37°C as described above and kept in culture for at least a week, before using them for an experiment.

Cell counting

10 µl of cell suspension were added to a Neubauer Chamber and covered with a cover glass. Cells that were within four counting grid squares were counted and this sum was multiplied by 2,500 to obtain the cell concentration of the suspension (cell number per ml).

4.2 Identification of EPOR knockout in H661 cells

A549 EPOR knockout cells were already generated and verified in a previous study [22]. The H661 knockout cell line was generated with the CRISPR/Cas9 System (Genescript, USA). To confirm the H661 EPOR knockout clone provided by Genescript we established monoclonal colonies by seeding single cells into 96 well plates. After growing a colony, cells were expanded in petri dishes with a 10 cm circumference. The genomic DNA of the different cell colonies was then isolated by incubating it in a homogenization buffer (50mM KCL, 10mM Tris-HCL (pH 8.3), 0.01mg/ml Gelatine, 0.045% Nonidet P-40, 0.045% Tween 20) with 50 µg/ml proteinase K (New England Biolabs) over night at 55°C. Proteinase K was heat-inactivated for 5-10 min at 95°C. The DNA extract was centrifuged for 5 min at 14860 rpm to pellet cellular debris and the supernatant was transferred into a new Eppendorf tube. Genomic human EPOR DNA was then amplified with a nested Polymerase Chain Reaction (PCR) using the Primers as seen in Table 1. The PCR products were purified using the QIAquick PCR Purification KIT (50) (QIAGEN). The purified PCR products were cloned into pGEM-T vector (Promega) following the supplier's instructions. The pGEM-T vectors were then transformed into NEB 5-α competent Escherichia coli (New England Biolabs) using the High Efficiency Transformation Protocol (New England Biolabs) [33] to isolate individual alleles. Successfully transformed E.coli formed colonies on a selection plate with ampicillin. The plasmid DNA was then collected using the QIAprep Spin Miniprep Kit

(QIAGEN) and sent to Microsynth for sequencing (Fig.1 A). The sequence reports were analyzed using BLAST NCBI [34]. The protein sequences that would result from these DNA sequences were determined using the ExPASy Translate Tool [35] (Fig.1 B). After identifying the clone with EPOR knockout on all four alleles in H661 we used western blotting to confirm the knockout on protein levels (Fig.1 C). Western Blot is a common technique used to separate and identify proteins. Through the use of polyacrylamide gel electrophoresis (PAGE), proteins that are loaded with sodium dodecyl sulfate (SDS) are negatively charged and are separated by molecular weight through the process of migration towards the anode. Following this separation step, the proteins are transferred to a membrane (electroblotting) which can then be incubated with the antibody against the protein of interest. The primary antibody will directly bind the protein of interest, whereas the secondary antibody binds to the primary antibody. Furthermore, the secondary antibody is linked to a horseradish peroxidase. This will catalyze a chemiluminescence reaction, when adding detection solution . [76]

Table 1 Sequences of the primers used for the nested PCR

Primer nested PCR	**Sequence**
PCR 1	F: 5'GGG GAC AGT AAG GCG AGA AAC T R:5'CAA ACA GCA GGG GAC ATA CGA G
PCR 2	F: 5'TCG GGG ATC TGC CAC TTA GA R: 5'CAC CAA GTC AGC CCC CTT AG

Figure 1

A

WT human EPOR DNA sequence

5'…CTGTGCTTCACCGAGCGGTTGGAGGACTTGGTG…3'
3'…GACACGAAGTGGCTCGCCAACCTCCTGAACCAC…5'

Allele 1 H661 EPORKO (-4 bp)

5'… CTGTGCTTCACCGAG----TGGAGGACTTGG…3'
3'…GACACGAAGTGGCTC----ACCTCCTGAACC…5'

Allele 2 H661 EPORKO (-10bp)

5'…CTTCACCGAGCGG----------TGGTGTGTTTCTGG…3'
3'…GAAGTGGCTCGCC----------ACCACACAAAGACC…5'

Allele 3 H661 EPORKO (-1bp)

5' …TTCACCGAGCGG-TGGAGGACTTGG…3'
3'…AAGTGGCTCGCC-ACCTCCTGAACC…5'

Allele 4 H661 EPORKO (+1bp)

5'…TCACCGAGCGGTGTGGAGGACTTGGTGT…3'
3'…AGTGGCTCGCCACACCTCCTGAACCACA…5'

B

WT Human EPOR protein sequence

MDHLGASLWPQVGSLCLLLAGAAWAPPPNLPDPKFESKAALLAARGPEELLCFTERLEDLVCFWEEAAS
AGVGPGNYSFSYQLEDEPWKLCRLHQAPTARGAVRFWCSLPTADTSSFVPLELRVTAASGAPRYHRVIH
INEVVLLDAPVGLVARLADESGHVVLRWLPPPETPMTSHIRYEVDVSAGNGAGSVQRVEILEGRTECVL
SNLRGRTRYTFAVRARMAEPSFGGFWSAWSEPVSLLTPSDLDPLILTLSLILVVILVLLTVLALLSHRR
ALKQKIWPGIPSPESEFEGLFTTHKGNFQLWLYQNDGCLWWSPCTPFTEDPPASLEVLSERCWGTMQAV
EPGTDDEGPLLEPVGSEHAQDTYLVLDKWLLPRNPPSEDLPGPGGSVDIVAMDEGSEASSCSSALASKP
SPEGASAASFEYTILDPSSQLLRPWTLCPELPPTPPHLKYLYLVVSDSGISTDYSSGDSQGAQGGLSDG
PYSNPYENSLIPAAEPLPPSYVACS

Allele 1 H661 EPORKO protein sequence

LRGAWSGRAWSAASGGGSC-PSCGLCRGRGTEGQEPWAPRGGGCIMDHLGASLWPQVGSL
CLLLAGAAWAPPPNLPDPKFESKAALLAARGPEELLCFTE**WRTWCVSGRKRRALGWARAT**
TASPTSSRMSHGSCVACTRLPRLVVRCASGVRCLQPTRRASCP-S

Allele 2 H661 EPORKO protein sequence

LRGAWSGRAWSAASGGGSC-PSCGLCRGRGTEGQEPWAPRGGGCIMDHLGASLWPQVGSL
CLLLAGAAWAPPPNLPDPKFESKAALLAARGPEELLCFTER**WCVSGRKRRALGWARATTA**
SPTSSRMSHGSCVACTRLPRLVVRCASGVRCLQPTRRASCP-

Allele 3 H661 EPORKO protein sequence

LRGAWSGRAWSAASGGGSC-PSCGLCRGRGTEGQEPWAPRGGGCIMDHLGASLWPQVGSL
CLLLAGAAWAPPPNLPDPKFESKAALLAARGPEELLCFTERWRTWCVSGRKRRALGWARA
TTASPTSSRMSHGSCVACTRLPRLVVRCASGVRCLQPTRRASCP-SCASQQPPALRDITV

Allele 4 H661 EPORKO protein sequence

LRGAWSGRAWSAASGGGSC-PSCGLCRGRGTEGQEPWAPRGGGCIMDHLGASLWPQVGSL
CLLLAGAAWAPPPNLPDPKFESKAALLAARGPEELLCFTERCGGLGVFLGGSGERWGGPG
QLQLLLPARG-A

C

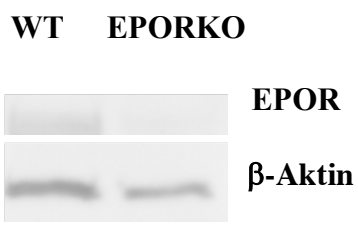


Fig. 1 Identifying EPOR knockout on four alleles in H661 cells.
A: Shown is the wild type DNA sequence of the human EPOR gene, as well as the four identified alleles harboring a functional EPOR knockout. Allele one contains a deletion of four base pairs (bp), the second allele a deletion of 10 bp, the third allele a deletion of one bp, and the fourth allele shows an insertion of one bp, marked as blue. **B:** Shows the wild type human EPOR protein sequence with the erythropoietin binding domain visualized in bold letters. Translating the CRISPR/Cas modified DNA sequences of EPOR into protein sequences shows the loss of the EPO binding domain in all four alleles. The protein encoding reading frames are highlighted in red. **C:** The loss of EPOR in H661 cells was verified on protein level using Western blot analysis using the GM1201 antibody. β-Aktin was used as a loading control.

To further characterize the A549 and H661 cells that were used, different proteins were measured via Western blotting.

4.3 Clonogenic Assay

Clonogenic Assays were performed in 25 cm^2 venti cap flasks (Corning, 430168) filled with 5ml of medium for radiation experiments, and on six well plates for chemotherapy experiments filled with 3ml of medium per well. The medium for A549 as well as H661 cells, contained 20% FCS instead of 10%. 250 A549 cells were seeded either into a 5 ml flask or a well of a six well plate. 750 H661 cells were seeded into a 5ml flask and 350 cells into a well of a six well plate. After seeding, the cells were incubated in normoxia for 24 hours. The cells were then either transferred into hypoxia, or remained in normoxia for another 24 hours. Next, the cells were then exposed to either chemotherapy or radiation as described below. After treatment, the cells were placed back into the hypoxic or normoxic chamber and incubated for six to eight days until colonies reached a size of approximately 50 cells. The cells were then stained over night with a solution containing 0.5% Crystal-Violet and 0.6% Glutaraldehyde. The solution was then removed, and the flasks or wells were washed with distilled water. After drying, all colonies with more than 50 cells were manually counted using a microscope by a blinded researcher. The number of treated cells was

normalized to the number of untreated control cells and results are shown in percentage of survival (%).

Treatments:

Radiation

The corning flask caps were tightly closed inside the hypoxic or normoxic incubators to prevent any gas exchange. The cells were transported to the radiation unit and exposed to a single dosage radiation (0,1,2 or 3 Gray (Gy)) with a 6 megavolt (MV) linear accelerator (Clinac iX, Varian, Palo Alto, CA, USA) using photons. Afterwards, they were placed back into the normoxic or hypoxic incubator, where the caps were re-opened inside the incubator for normal gas exchange.

Chemotherapy

Cells were treated either with Gemcitabine (TEVA Pharma) or Taxol (1152601, Bristol-Myers Squibb). Concentrations of 0,1,2,3,4 and 5 ng/ml of chemotherapeutics were used.

Wildtype and EPOR knockout preconditioned medium

A549 and H661 Wild type (WT) and EPOR knockout (EPORKO) cells were split in a ratio of 1 to 10, seeded onto 10 cm plates and incubated for 72 hours with 20 % FCS medium. Plates were then incubated either in hypoxia or normoxia for 24 hours. The preconditioned medium was then collected in 50ml Falcon tubes and centrifugated at 180 g for 5 min to remove cell debris. The supernatant, i.e. the debris free preconditioned medium, was collected. Pre-seeded (24 h) A549 or H661 cells were incubated with the preconditioned medium for 24 hours. Afterwards, they were then treated or not with either 3 Gy radiation or 1.5 ng/ml (A549 cells) as well as 2 ng/ml (H661) Gemcitabine and incubated until colonies grew.

Boiling the preconditioned medium

As a negative control, parts the aforementioned preconditioned medium were boiled at 95°C in a water bath for 10 min. After cooling

down, the medium was applied to A549 cells as described above. The cells were treated with 1.5ng/ml Gemcitabine 24 hours later and incubated until colonies grew.

Exosome Extraction

Preconditioned medium of normoxia or hypoxia exposed A549 cells, which contained 20% of exosome depleted FCS (Thermofisher, Cat A2720803), were centrifuged to remove the remaining cell debris. Exosomes were extracted using the Exosome Purification Kit (EX01-Exo Spin- Cell Guidance Systems) and diluted in fresh 20% FCS medium. Pre-seeded (24 h) A549 cells were incubated with exosome containing medium. They were then treated with 0ng/ml or 1.5ng/ml Gemcitabine 24 h later, and incubated until colonies grew.

Recombinant Human Erythropoietin Treatment

A549 cells were seeded and 24 or 48 hours afterwards, cells were treated with 5 units/ml of rhEPO (Recormon; Roche). 24, 2, and 0.5 hours after EPO treatment, cells were irradiated with 0 or 3 Gray and incubated until colonies grew.

Soluble EPOR and EPOR Blocking Peptide EMP9 treatment

A549 wild type cells were seeded and incubated for 24 h in normoxia. Right before the cells were placed in either normoxia or hypoxia, 0.5µg/ml of soluble EPOR [77] (NS0-307-ER, R&D Systems) or 5µg/ml of EMP9 [78] with the sequence (N to C) GGTYSCHFAPLTWVCKPQGG (SP190977, Biomatik) was applied and cells were incubated for 24 hours. Afterwards they were irradiated with 3 Gy or not (controls) and incubated at normoxia or hypoxia until colonies grew.

Growth Factor treatment

0.05µg/ml Stem Cell Factor (NBP2-35256, Novus Bio), 12.5µg/ml Thrombospondin-1(NS0- 3074-TH, R&D Systems), or 1.0µg/ml Ephrin B2 (10881-HCCH, Sino Biological) was applied to normoxia-exposed A549 cells

24 h after seeding. 24 h after incubation with growth factors, the cells were treated with 0ng/ml or 1.5ng/ml Gemcitabine or not (controls) and incubated in normoxia until colonies grew.

4.4 Erythropoietin activity in Mice

In this study we verified the activity of EPO used for our *in vitro* assays. We used eight female mice with a mixed background comprised of FVB, C57Bl/6 and 129S, that were 36 weeks of age. The mice were housed at 22±5 °C in a 12h light/dark cycle and fed rodent chow and water freely. The mice were intraperitoneally injected with either 100 μl 300units/kg of rhEPO (Recormon; Roche) or 100 μl saline three times a week (Monday, Wednesday, and Friday) for two weeks. Three days after the last injection, the mice were euthanized by carbon dioxide (CO_2). After opening the chest cavity to expose the heart, 0.5-1ml of blood were collected by punctuating the right ventricle with a 1ml G21 syringe that contained heparin on the inside. The hematocrit was determined by filling capillaries with blood, centrifuging the capillaries for 5 min at 120rpm in the microcentrifuge (HETTICH), and calculating the ratio of volume of the red blood cell pellet to the entire blood volume (%). The hemoglobin was measured using the ABL800 (Radiometer RSCH GmbH).

4.5 RNA Extraction, cDNA Synthesis and Real-time PCR

To extract the RNA from *in vitro* cultivated cells, the ReliaPrep RNA Cell Miniprep System was used. Briefly described, 1- Thioglycerol was freshly added to the LBA buffer that was supplied with the kit before the RNA extraction. The cells on 6 well plates were washed with cold PBS. After aspirating the PBS, cells were incubated with 250µl of the 1-Thioglycerol LBS mix. The cells were scraped off the plates and the cell suspension was transferred into an Eppendorf tube, which was kept on ice. To complete the cell lysis, the cells were passed through a 21G syringe several times and the lysate was centrifugated at 16 g for 5 min. The supernatant was transferred to a ReliaPrep Minicolumn in a collection tube, kept at room temperature, and centrifuged with the same conditions as above. After the flow through was discarded, an RNA wash solution was applied to the column, followed by another centrifugation step.

Again, the flow through was discarded and the columns were incubated with DNaseI mix, which was prepared following the instructions in the protocol, for 15 min at room temperature. Afterwards, a column wash solution was directly applied without prior centrifugation. The wash solution was removed by centrifugation and the process was repeated once. To eluate RNA from the columns, 30µl of RNA free water was added to the columns and the columns were centrifugated. The RNA quantity was assessed by measuring its absorption at 260 nm using the NanoDrop 2000 Spectrophotometer and RNA quality was determining the ratio of 260/280 as well as 260/230. The RNA was either stored at -80°C or directly transcribed into cDNA.

To transcribe the RNA into cDNA, 10µl of RNA (100ng/µl) was incubated with 1µl of Oligo dT (10µM; Promega) for 5 minutes at 65°C. The mix was placed on ice for another 5 minutes. The samples were then incubated with 15µl of master mix (5.57 µl H_2O, 5 µl 5x Reaction Buffer for RT (Thermo Fischer), 2.5 dNTPs (15 mM each, Thermo scientific), 1 µl RNasin (Promega), 0.25 µl 100x BSA (New England Biolaps) and 0.5 µl Revert Aid Reverse Transcriptase (Thermo scientific) for 2 hours at 42°C. The reaction was inactivated by incubating the samples at 65°C for 10 minutes. To obtain a cDNA concentration of 5ng/µl, 175 µl of H_2O were added. Samples were stored at -20 °C or directly used for real time PCR.

For real time PCR a master mix with 5µl PowerUp SYBR Green master mix (ThermoFisher, Cat A25743), 1µl Primer forward (10 µM), 1µl Primer reverse (10 µM) and 1µl of SYBR H_2O was prepared on ice and 7.5 μl of the mix were pipetted into a well MicroAmp Fast Optical 96-Well Reaction Plate (Applied Biosystems by life technologies). After adding 2 µl of cDNA (5ng/µl), the plate was sealed with Optical Adhesive Covers (Applied Biosystems by Life Technologies), and centrifuged for a few seconds at 2500 x g, before being placed in Thermocycler ABI7500 Fast (Applied Biosystems). The Amplification profile was as follows: 50° C for 2 min; 95° C for 10 min; 40 cycles at 95° C for 30 sec and 60° C for 40 sec, followed by melt curve analysis. The samples were run in duplicates and the results obtained were normalized to β-actin (reference gene) and fold changes were calculated using the ΔΔCt method [79] with the following formulae:

$\Delta Ct = Ct_{Target} - Ct_{Reference}$

$\Delta\Delta Ct$= (CtTarget – CtReference) sample – (CtTarget – CtReference) calibrator

Fold change= $2^{\Delta\Delta Ct}$

The primers used for real time PCR were designed using the Primer3 Software [39] [40] and ordered from Microsynth. Primer quality was assessed by the melting Curve analysis after the real time PCR run, as well as by gel electrophoresis to determine the size and purity of the product. The primer sequences are shown in Table 2.

Table 2 Primer sequences used for RT- PCR.

Target Gene	**Primer Sequence**
Human β-Aktin	Fwd: 5' CTGGAACGGTGAAGGTGACA 3' Rev: 5' AAGGGACTTCCTGTAACAACG 3'
ATG 5	Fwd: 5' ATGTGCTTCGAGATGTGTGG 3' Rev: 5' CATTTCAGTGGTGTGCCTTC 3'
ATG 6	Fwd: 5' AGGTTGAGAAAGGCGAGACA 3' Rev: 5' AGGACACCCAAGCAAGACC 3'
ATG 7	Fwd: 5' TGGAACAAGCAGCAAATGAG 3' Rev: 5' AGACAGAGGGCAGGATAGCA 3'
ATG 12	Fwd: 5' AGTAGAGCGAACACGAACCA 3' Rev: 5' GGGAAGGAGCAAAGGACTGA 3'
CD131	Fwd: 5' AATGTGTGGATGTGAGACTGAGG 3' Rev: 5' GCCATAGAGAAAGCAAGGTAACAAA 3'
H661 EPOR	Fwd: 5' GGGGACAGTAAGGCGAGAAAC 3' Rev: 5' CAAACAGCAGGGGACATA CGA 3'
YWHAZ (14-3-3 Zeta Protein)	Fwd: 5' CCGTTACTTGGCTGAGGTTG 3' Rev: 5'AGTCTGATAGGATGTGTTGGTTG3'
CLU (Clusterin)	Fwd: 5' GAGACCAGGGAATCAGAGACA 3' Rev: 5' TTTCAGGCAGGGCTTACACTC
ICOSLG (B7RP1) (inducible T cell costimulator ligand)	Fwd: 5' CCACTCCAGACCTCCCTTCCTC 3' Rev: 5' AGCCCAGCCCGAGAACAAAC 3'

4.6 Western Blot

To analyze the proteins of *in vitro* cultivated cell lines, the cells were lysed with RIPA Buffer (20 mM Tris, 150 mM Sodium chloride, 1% Triton X-100, 1% Na-deoxycholate, 0.1% SDS) directly on the plate and the lysate was kept in Eppendorf tubes on ice. To determine the protein concentration, we used the Pierce Bicinchoninic Acid (BCA) Protein. Protein lysates were incubated with a sample buffer containing 5% SDS and 5 % beta-mercapto-ethanol, an SDS- PAGE gel was casted (Table 3), and the protein lysates were loaded on the SDS-PAGE gel.

Table 3 Ingredients for the SDS-PAGE Mix Western Blot Gel

Layer	**Separation**	**Collecting Gel**
Dest H_2O (ml)	6.8	3.6
1.5M Tris, pH 8.8 (ml)	3.0	-
0.5M Tris, pH 6.8 (ml)	-	0.5
Acrylamid Stock (ml) 30% Acrylamid/Bis 29:1	5.0	0.8
10% SDS (µl)	150	50
10% APS (µl)	50	35
TEMED (µl)	7.5	10

The gel was run at constant 15mA for 15 min, and then at constant 25mA for 60 minutes (Bio- Rad Power Pac 1000). The separated proteins were transferred to a nitrocellulose blotting membrane (GE Healthcare Life Science) at 1000 mA for about 2 hours. After the transfer, the membrane was blocked using 5% milk (Rapilait, Migros, Schweiz) in Tris buffered saline (pH 7.5) containing 0.3% Triton X-100 (TBST). The following primary antibodies were diluted in 5% milk: rat anti-EPOR

1:200 (GM1201; Adlevon), mouse anti EPOR BCO-4B5-C9 1:500 (not available anymore; Adlevon), rabbit Vimentin (R28) (3932S; Cell Signaling) 1:500, mouse E-cadherine (ab1416; Cell Signaling) 1:500, Snail (C15D3) Rabbit mAb (3879S; Cell Signaling) 1:500, rabbit anti Cyclin D1(2922; Cell Signaling) 1:200, and mouse mAb Cyclin E (4129; Cell Signaling) 1:200. The membranes were incubated in antibody solutions over night at 4°C. After washing them three times with TBST, the membranes were incubated with HRP conjugated secondary antibodies, which were diluted in 5% milk in TBST: 1:5000; goat anti-rat (sc-2032; Santa Cruz), donkey-anti-rabbit (NA934V; Amersham), and goat-anti-mouse (Santa Cruz sc-2031). To visualize the protein bands, the membranes were incubated with Super Signal West Femto Maximum Sensitivity Substrate (Thermo Scientific) for a few minutes and chemiluminescent signals were recorded with FUJIFILM Intelligent Darkbox Las-300.

5. Results

5.1 Hypoxia-inducible resistance to chemo- and radiotherapy is EPOR-dependent.

To analyze the role of EPOR on hypoxia-induced resistance to chemo- and radiotherapy, we used the previously described A549 WT and EPORKO as well as our newly established H661 WT and EPORKO lung cancer cell lines. Both cell lines responded adequately to hypoxia as exemplified by the hypoxia-induced expression of *Egln1* (supplemental, Fig.1). Additionally, A549 WT and EPOR as well as H661 WT and EPOR formed colonies in a clonogenic assay. We used this assay to determine the survival of normoxia and hypoxia exposed A549 WT (Fig.2 A) and EPORKO (Fig.2 B) cells at different concentrations of Taxol. Survival of both, A549 WT and EPOKO cells, decreased in a dose-dependent manner under normoxia as well as hypoxia. However, hypoxia exposed A549 WT but not EPORKO cells had a better survival rate than normoxia exposed cells. When A549 WT cells were incubated with 1.5 ng/ml Taxol the survival rate increased from 28% at normoxia to 47% at hypoxia ($p<0.05$). However, the survival rate of A549 EPORKO cells decreased from 30% at normoxia to 18% at hypoxia (Fig.2 A and B). This indicates that EPOR WT but not EPOR deficient A549 cells were protected from Taxol by hypoxia exposure. In addition, we analyzed the response of H661 lung cancer cells to Taxol to confirm that the effect is not only cell line-specific. H661 cells showed a similar pattern: Taxol-exposed EPOR wild type cells displayed better survival rates under hypoxia than under normoxia (Fig.2 C). This effect was lost in EPORKO cells, which were not protected from Taxol under hypoxia (Fig.2 D).

In a further experiment, we exposed normoxic and hypoxic H661 lung cancer cells with and without EPOR expression to different concentrations of Gemcitabine, which is a common therapeutic used for advanced lung cancer patients [80]. When H661 WT cells were incubated with 3ng/ml Gemcitabine, the survival rate increased from 21% at normoxia to 33% at hypoxia ($p<0.05$). However, the survival rate of H661 EPORKO cells did not differ between normoxia and hypoxia (Fig.2 E and F). This shows that EPOR WT but not EPOR deficient H661 cells were protected from Gemcitabine by hypoxia exposure. Lastly, we analyzed the response of H661 lung

cancer cells to radiation. Radiation-exposed EPOR wild type cells displayed better survival rates under hypoxia than under normoxia (Fig.2 G). At a radiation dose of 3 Gy, the H661 WT cells showed a survival rate of 58% under hypoxia, whereas under normoxia the survival rate was only 40% ($p<0.05$). This effect was lost in EPORKO cells, which were not protected from Radiation under hypoxia (Fig.2 H).

Our data suggest that hypoxia exposure induces a general resistance to chemo- and radiotherapy in lung cancer cells and that the resistance depends on the expression of EPOR in lung cancer cells.

Figure 2

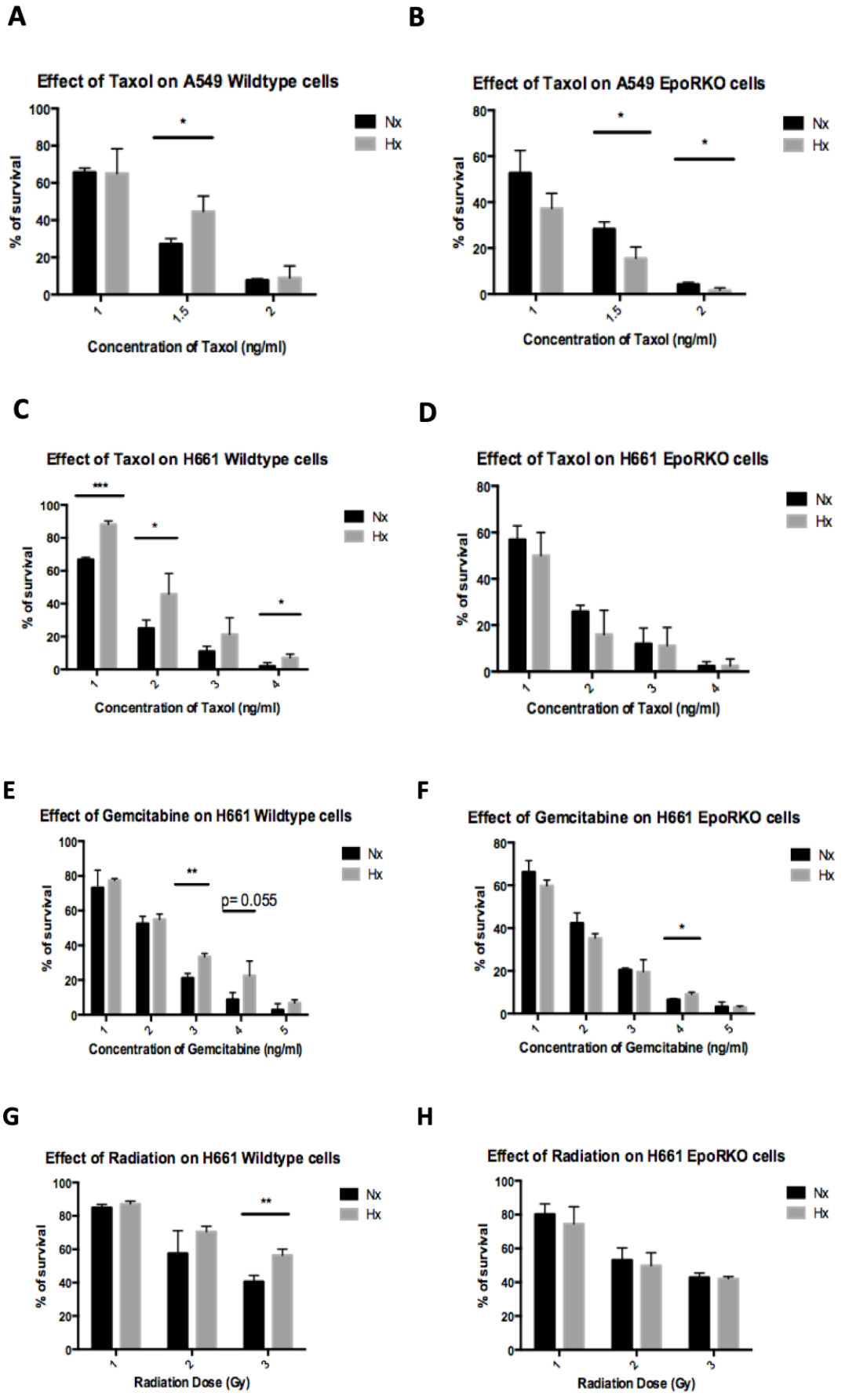

A
Effect of Taxol on A549 Wildtype cells
% of survival
Nx
Hx
Concentration of Taxol (ng/ml)
B
Effect of Taxol on A549 EpoRKO cells
% of survival
Nx
Hx
Concentration of Taxol (ng/ml)
C
Effect of Taxol on H661 Wildtype cells
% of survival
Nx
Hx
Concentration of Taxol (ng/ml)
D
Effect of Taxol on H661 EpoRKO cells
% of survival
Nx
Hx
Concentration of Taxol (ng/ml)
E
Effect of Gemcitabine on H661 Wildtype cells
p= 0.055
% of survival
Nx
Hx
Concentration of Gemcitabine (ng/ml)
F
Effect of Gemcitabine on H661 EpoRKO cells
% of survival
Nx
Hx
Concentration of Gemcitabine (ng/ml)
G
Effect of Radiation on H661 Wildtype cells
% of survival
Nx
Hx
Radiation Dose (Gy)
H
Effect of Radiation on H661 EpoRKO cells
% of survival
Nx
Hx
Radiation Dose (Gy)

Fig. 2 EPOR dependent hypoxia-inducible resistance to chemotherapy and radiotherapy in A549 and H661 lung cancer cells

A549 and H661 lung cancer wildtype cells (WT) and EPO receptor knockout cells (EPORKO) were incubated either in normoxia (21% O_2, black bar; Nx) or hypoxia (1% O_2, gray bar; Hx) and treated with different concentrations of Taxol, Gemcitabine or Radiation. The survival rate was analyzed by clonogenic assay and is shown relative to untreated controls of the respective EPOR expressing or deficient cell line in percent (%).

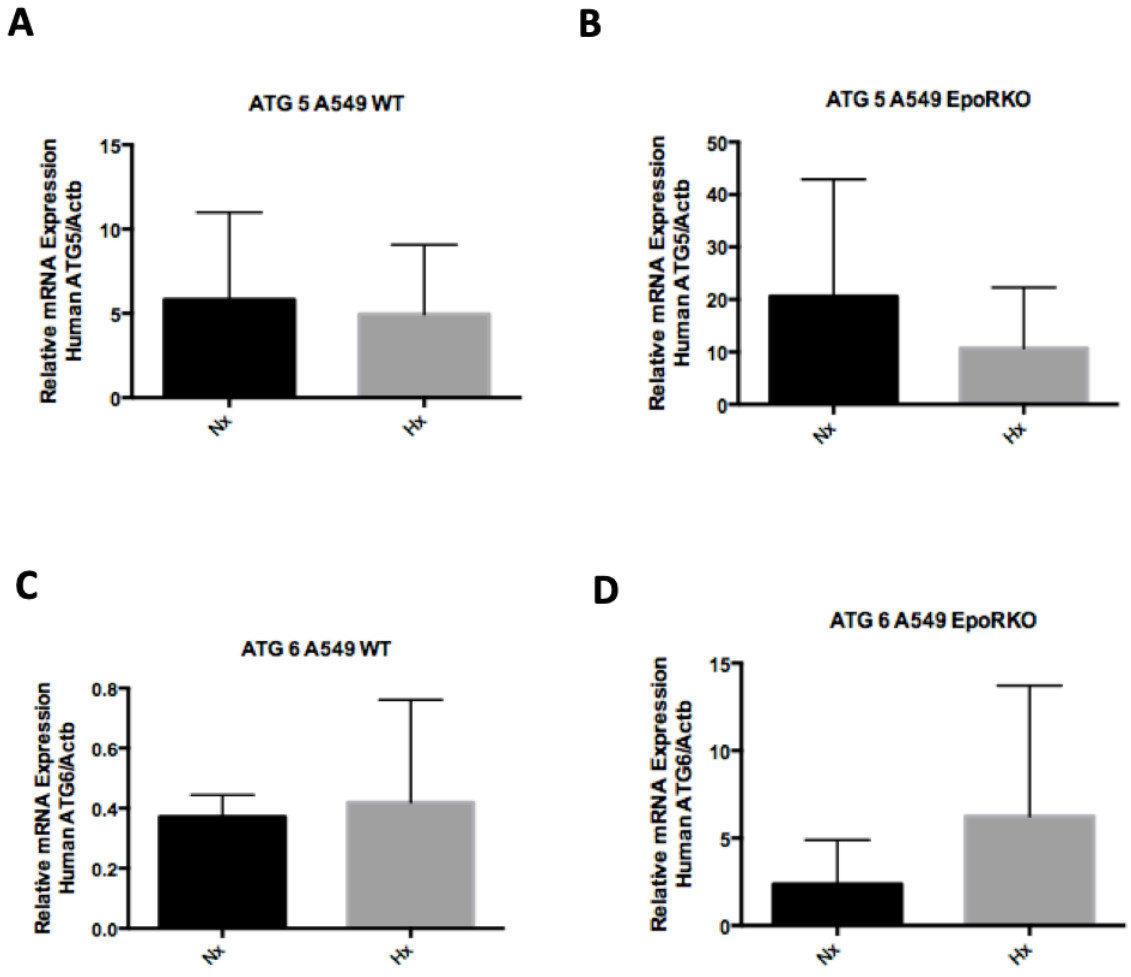
Panel **A** shows A549 WT and panel **B** shows A549 EPORKO lung cancer cells exposed to an increasing concentration of Taxol (X-axis) under normoxic (black) and hypoxic (gray) conditions (n=3). **C** and **D** show H661 WT (**C**) and EPORKO (**D**) lung cancer cells exposed to an increasing concentration of Taxol under normoxic (black) and hypoxic (gray) conditions (n=3). Panel **E** shows H661 WT, and Panel **F** shows H661 EPORKO cells that are exposed to an increasing concentration of Gemcitabine under normoxic (black) and hypoxic (gray) conditions (n= 3). **G** illustrates H661 WT and **H** illustrates H661 EPORKO cells that are exposed to an increasing dose of radiation under normoxic (black) and hypoxic (gray) conditions (n=3). Shown is the percentage of survival (Y-axis) normalized to untreated WT and EPOR knockout cells in %. A Students t-test was performed for statistics. (Mean ± SD; n=3-4; *= $0.01 \leq p < 0.05$, **= $0.001 \leq p < 0.01$, *** = $p < 0.001$

5.2 Autophagy is not involved in EPOR-mediated protection from chemo- and radiotherapy

Radiotherapy increases DNA damage and induces DNA repair mechanisms [84]. Thus, it is reasonable to assume that hypoxia-driven cell protection from radiation involves DNA repair mechanisms to prevent cell death [85]. However, a previous study in our lab showed that neither DNA damage nor repair mechanisms are involved in the hypoxia-induced protection of A549 lung cancer cells from radiation [22]. Radiation-induced autophagy often protects cells from cytotoxic stimuli [86], [127]. Initially it can prevent or at least delay the formation of tumor by protecting the cell from potentially damaging species that might lead to mutational and carcinogenic damage. However, once tumor formation has progressed, autophagy can protect the tumor cell from environmental injury. In radio- and chemotherapy, the induction of autophagy is frequently thought to perform an additional cytoprotective function by preventing cell death through apoptosis [86]. Because EPO can suppress apoptotic cell injury through autophagy [87], we compared the mRNA expression of the relevant autophagy genes ATG 5, 6, 7, and 12 of normoxia and hypoxia exposed A549 WT and EPORKO cells (Fig.3). The expression of ATG 5, a key protein, is reduced 1.2 times ($p>0.05$) in both, hypoxia exposed A549 WT as well as EPORKO cells (Fig.3, A; B, respecitively). Furthermore, the expression of ATG 6 did not differ between normoxia and hypoxia exposed A549 WT cells (Fig.3 C). Additionally, ATG 6 expression increased three times ($p>0.05$) in A549 EPORKO cells under hypoxic conditions (Fig.3 D). ATG7 expression was increased in hypoxic conditions in both, A549 WT (Fig.3 E) and A549 EPORKO (Fig.3 F) cells. In the A549 WT cells, the increase was 2.16-fold ($p>0.05$), and in the EPORKO it was even 8-fold ($p>0.05$).

The expression of ATG12 increased 1.22-fold ($p>0.05$) in the A549 WT cells under hypoxic conditions (Fig.3 G), whereas ATG12 gene expression could not be measured in hypoxia exposed A549 EPORKO cells (Fig.3 H). In summary, the data on autophagy gene expression were mixed and show no clear indication of an EPOR dependent activation of autophagy in lung cancer cells under hypoxia. Thus, we conclude that the EPO-EPOR driven induction of autophagy is not involved in the hypoxia-induced protection of A549 lung cancer cells from chemo- and radiotherapy.

Figure 3



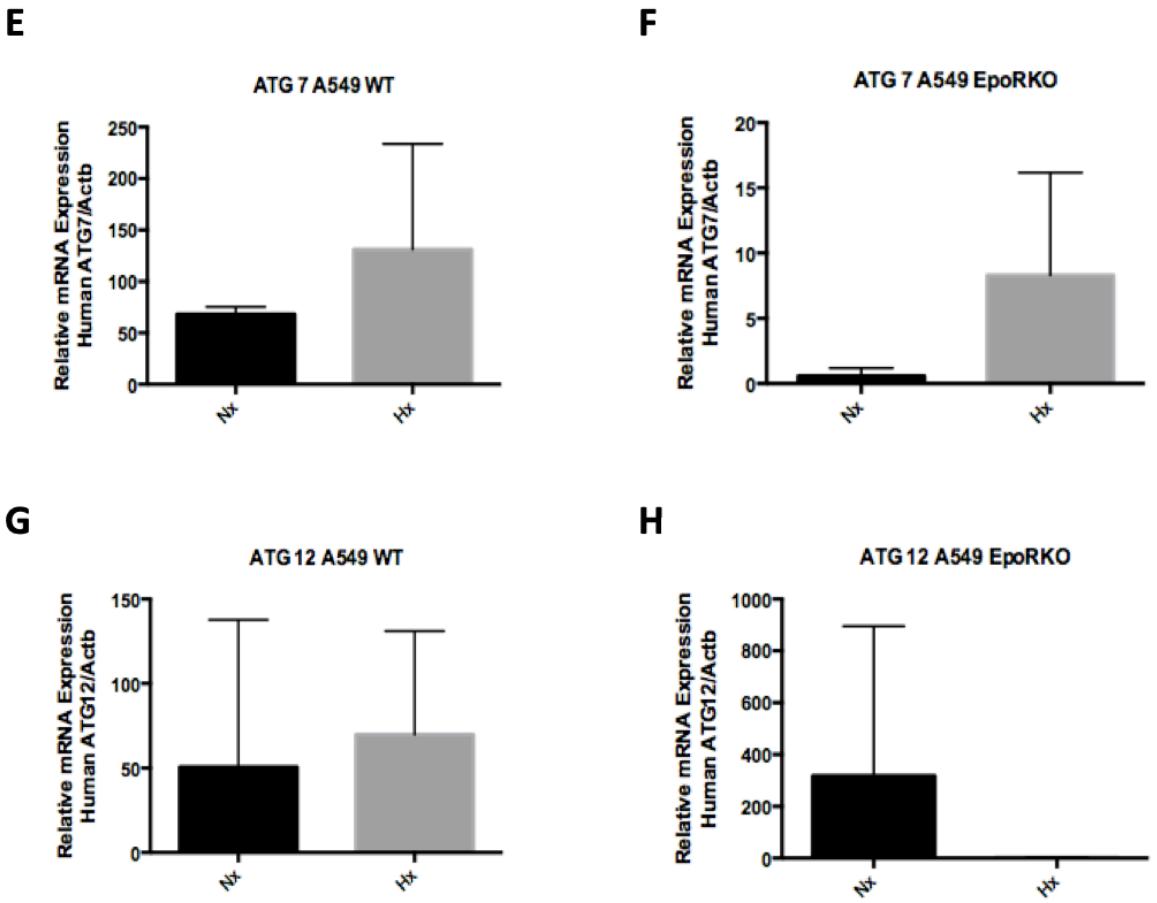


Fig.3 Autophagy- related Genes (ATG) Real Time PCR Results
Real Time PCR was performed using cDNA of the A549 lung cancer wildtype cells (WT) and EPO receptor knockout cells (EPORKO) that were cultured in normoxic (21% O_2; black bars) and hypoxic (1% O_2; gray bars) conditions. The results obtained were normalized to β-actin (calibrator) and fold changes were calculated using the ΔΔCt method. The samples were run in duplicates. Panels **A** and **B** show *ATG5* expression of A549 WT cells (**A**) (n=3), and A549 EPORKO cells (**B**) (n=3). Panels **C** and **D** show *ATG6* expression of A549 WT cells (**C**) (n=3), and A549 EPORKO cells (**D**) (n=3). Panels **E** and **F** show *ATG7* expression of A549 WT cells (**E**) (n=3), and A549 EPORKO cells (**F**) (n=3). Panels **G** and **H** show *ATG12* expression of A549 WT cells (**G**) (n=3), and A549 EPORKO cells (**H**) (n=3). A Students t-test was performed for statistics. (Mean ± SD; n=3-4;
*= $0.01 \leq p < 0.05$, **= $0.001 \leq p < 0.01$, *** = $p < 0.001$

5.3 Erythropoietin is not required for EPOR-mediated protection from chemo- and radiotherapy

As shown before by N. Jänicke [22] and in Figure 2, EPOR contributes to hypoxia induced therapy resistance. Thus, it seems plausible that the ligand of EPOR, erythropoietin (EPO), induces protection from chemo- and radiotherapy in lung cancer cells, which has been discussed since the early 2000s [2], [7], [12], [13]. On the other hand, A549 cells were reported not to respond to EPO [124]. To test the role of EPO in hypoxia-induced protection of lung cancer cells, we incubated the A549

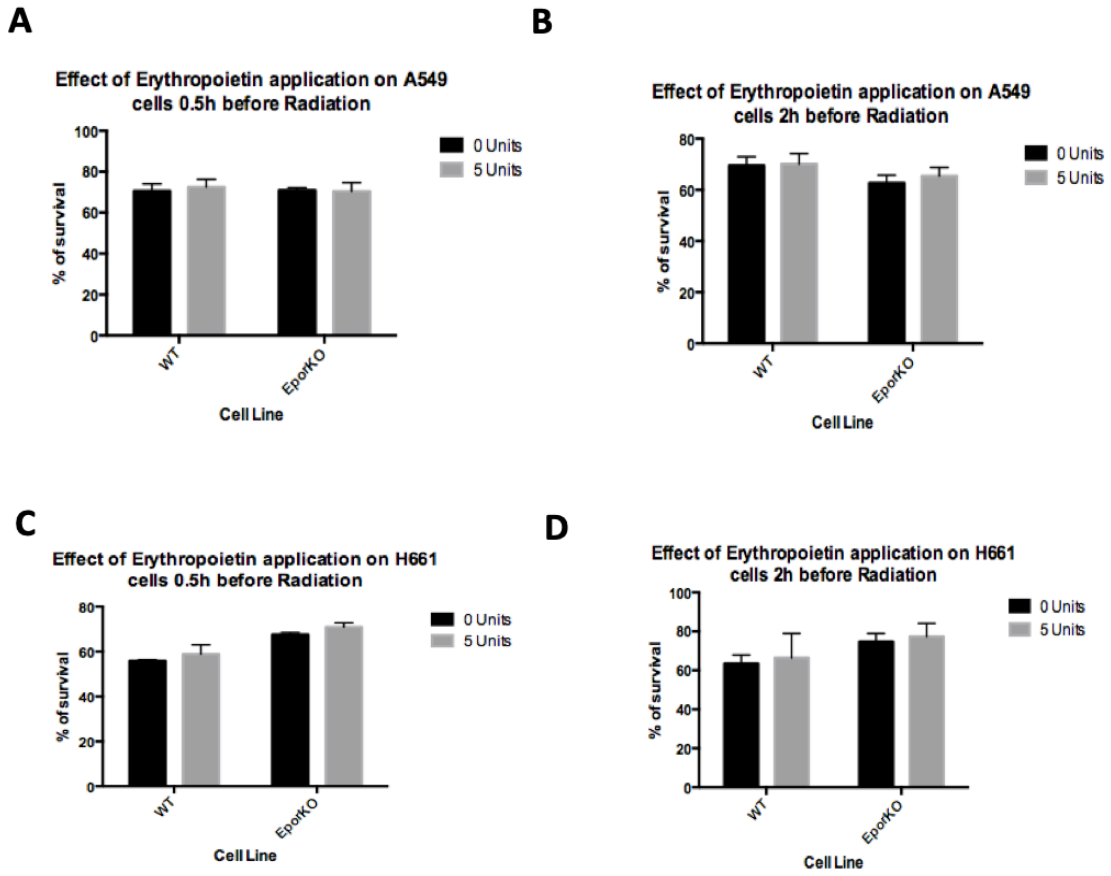
WT cells with 1 and 2.5 u/ml EPO under normoxic conditions prior to radiation with 3 Gy (supplemental Fig.3). However, EPO treatment did not protect A549 WT cells from radiation. We further incubated WT and EPORKO A549 and H661 lung cancer cells with a higher dose of 5 u/ml EPO under normoxic conditions, 0.5 and 2 hours prior to radiation with 3 Gy. Our data show that EPO treatment did neither protect A549 WT nor EPORKO cells from radiation, when EPO was applied 0.5 (Fig.4 A) or 2 hours (Fig.4 B) prior to radiation. Similarly, H661 WT and EPORKO cells were not protected by EPO when treated 0.5 (Fig.4 C) or 2 hours to prior radiation (Fig.4 D). Next, we tested if lung cancer cells might require both, hypoxia exposure and EPO stimulation, to survive anti-cancer treatment. Therefore, we incubated A549 WT cells either in normoxia or hypoxia with and without 5 u/ml EPO and treated them with 1.5ng/ml Gemcitabine. As seen before, A549 WT cells showed increased survival at hypoxia. However, EPO administration did not further improve survival of A549 WT cells in neither normoxia nor in hypoxia (Fig.4 E).

To verify that EPO used for *in vitro* experiments was active, we injected mice and analyzed their hematocrit and hemoglobin. Injection with 300 u/kg EPO increased hematocrit from 48% to 77% ($p<0.001$), as well as hemoglobin from 132 to 227g/l ($p<0.001$) (Fig.4. F). Thus, we concluded that the EPO was active and that the hypoxia-induced protection of lung cancer cells from chemotherapy and radiation was independent of administered EPO.

A549 lung cancer cells hardly express endogenous *Epo* mRNA levels under normoxia and hypoxia [22]. However, because cells *in vitro* are cultivated in 10-20% heat-inactivated FCS, it might be possible that bovine EPO in FCS is sufficient to bind and fully activate the human EPOR in *in vitro* cultivated lung cancer cells. Thus, we incubated A549 (Fig.4 G) as well as H661 (Fig.4 H) WT cells with 0.5ug/ml of soluble EPOR (sEPOR) 24 hours prior to radiation. Although it is not clear if bovine EPO can activate human EPOR [57], we assume that, if bovine EPO binds to human EPOR on cancer cells, it can also be sequestered by sEPOR. As shown above, exposure to hypoxia increased the survival of irradiated A549 and H661 WT cells from approximately 60% to 80% ($p>0.05$). However, the incubation with sEPOR did not prevent the hypoxia-driven protection from radiation, which suggests that EPO (either endogenously produced by the hypoxia-exposed cancer cells or residual bovine EPO in FCS) is not required to activate EPOR in lung cancer cells. This was further

supported by using the EPOR antagonizing peptide EMP9 [81]. A549 WT cells were either kept at normoxia or hypoxia and radiated with 3 Gy. The survival rate of hypoxia exposed cells increased from 61% to 79% ($p>0.05$) but EMP9 did not prevent hypoxia-induced protection from radiation (Fig.4 I).

Figure 4



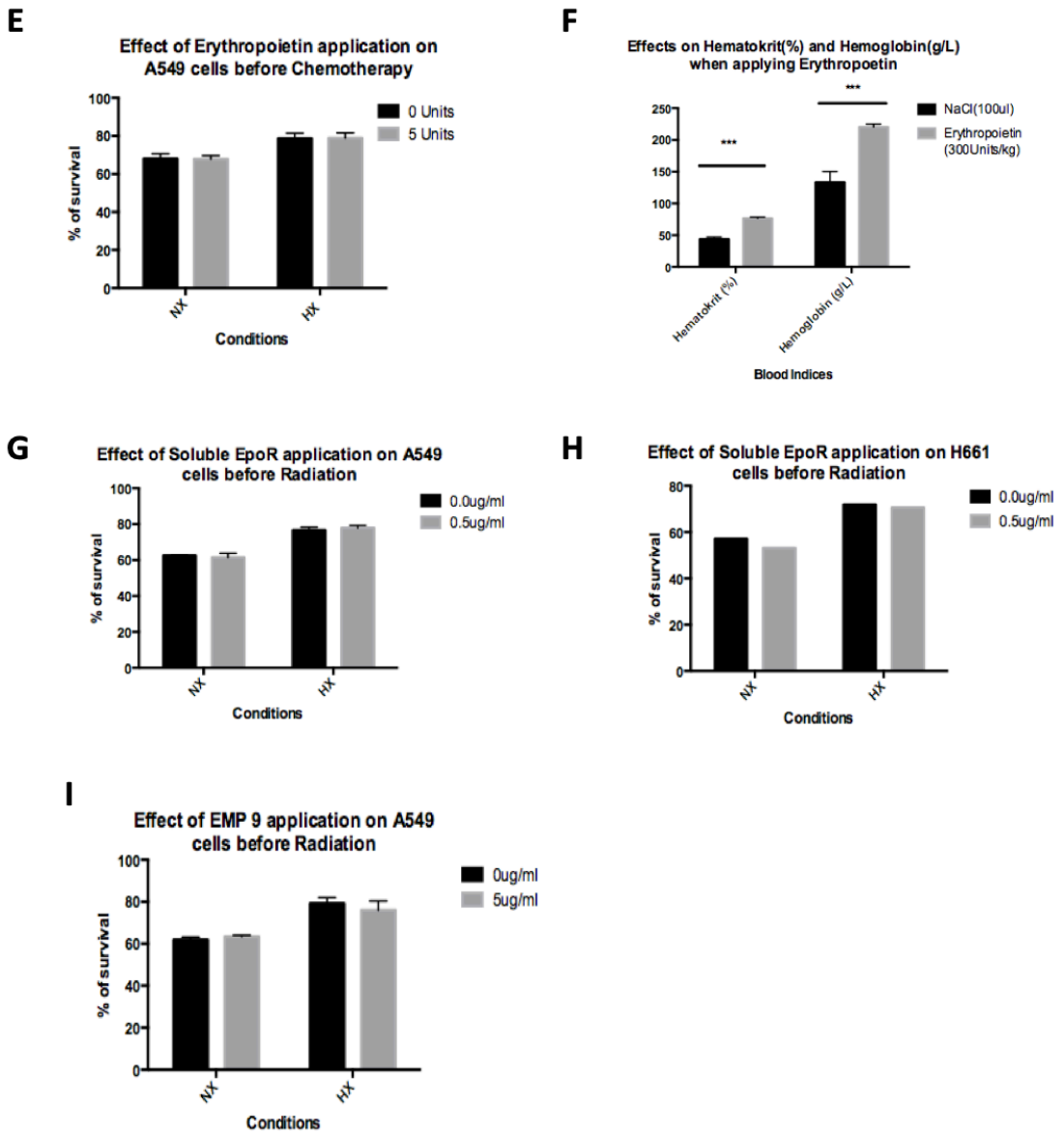


Fig. 4 Hypoxia-induced therapy resistance against Gemcitabine and radiation is EPO independent.
A549 and H661 lung cancer wildtype cells (WT) and EPO receptor knockout cells (EPORKO) were incubated either in normoxia (21% O_2, Nx) or hypoxia (1% O_2, Hx) and treated with 1.5ng/ml Gemcitabine or 3 Gy radiation. The survival rate was analyzed by clonogenic assay and is shown in percent (%) to untreated controls of the respective EPOR expressing or deficient cell lines. Panels **A** and **B** show survival of in normoxia (21 %) cultivated A549 WT and EPORKO in response to 3 Gy radiation. Either 0.5h (**A**) (n=3) or 2h (**B**) (n=3) prior to radiation, cells were incubated with 5 u/ml EPO (gray bars) or not (black bars). Panels **C** and **D** show survival of in normoxia (21 %) cultivated H661 WT and EPORKO in response to 3 Gy radiation. Either 0.5h (**C**) (n=3) or 2h (**D**) (n=3) prior to radiation, cells were incubated with 5 u/ml EPO (gray bars) or not (black bars). Panel **E** shows survival of in normoxia (21%) or hypoxia (1%) cultivated A549 WT cells in response to 1.5ng/ml Gemcitabine after the cells were incubated with 5 u/ml EPO (gray bars) or not (black bars) (n=3). Panel **F** displays the efficacy of the EPO used. It shows the increase in hematocrit (%) and hemoglobin (g/L) of mice treated with EPO (gray bar), or with NaCl (black bar) (n=4). Panels **G** and **H** show survival of in normoxia (21 %) or hypoxia (1%) cultivated A549 WT (**G**) (n=3) and H661 WT (**H**) (n=1) in response to 3 Gy radiation. Cells were incubated with 0.5ug/ml of sEPOR (gray bars) or not (black bars). Panel **I** shows survival of the A549 WT cells cultivated in normoxia (21%) or hypoxia (1%) in response to 3 Gy radiation (n=3). Either 5ug/ml EMP9 were applied (gray) or not (black). A Students t-test was performed for statistics. (Mean ± SD; n=3-4; *= $0.01 \leq p < 0.05$, **= $0.001 \leq p < 0{,}01$, *** = $p < 0.001$

5.4 Hypoxia-induced protection is mediated by a secreted factor

Hypoxia protects A549 and H661 lung cancer cells from radiation as well as Taxol and Gemcitabine. We showed that this protection requires the expression of EPOR. However, it does not require EPO. Because cytokine receptor activation, i.e. EGFR, has been suggested to be non-ligand induced [82], it is possible that EPOR is activated by hypoxia, however non-ligand induced. To test if cell secreted factors (i.e. paracrine activation) are required to mediate protection from radiation and chemotherapy, we collected the medium of A549 and H661 cells either exposed to normoxia or hypoxia (preconditioned medium). We observed that hypoxia preconditioned medium protects A549 and H661 lung cancer cells from Gemcitabine and radiation in a clonogenic assay: The survival rate of Gemcitabine-treated A549 WT cells increased from 51% in normoxia preconditioned medium to 69% in hypoxia preconditioned medium ($p<0.01$) (Fig.5 A). In contrast A549 EPORKO cells showed no increased survival rate, when incubated in a hypoxic preconditioned medium (Fig.5 A). Similarly, the survival rate of Gemcitabine-treated H661 WT cells but not of H661 EPORKO cells increased from 52% in normoxic preconditioned medium to 64% in hypoxic preconditioned medium ($p<0.01$) (Fig.5 B). Furthermore, the survival rate of 3 Gy radiated A549 (Fig.5 C) and H661 (Fig.5 D) WT but not EPORKO cells increased from 52% and 53%, respectively in normoxic preconditioned medium, and to 62% and 64%, respectively, in hypoxic preconditioned medium ($p<0.05$). These data suggest that a factor, which protects cancer cells via EPOR, is produced and is secreted by hypoxia exposed cancer cells. To test if the secreted factor is a protein, we boiled the normoxic and hypoxic preconditioned medium to denaturate proteins. While hypoxic preconditioned medium increased the survival rate of Gemcitabine treated A549 WT cells from 76% in normoxic preconditioned medium to 83% ($p<0.01$), no difference between Gemcitabine-treated cells incubated in boiled normoxia or hypoxia preconditioned medium was observed (Fig.5 E). Thus, we conclude that activation of EPOR for protecting lung cancer cells from chemo- and radiotherapy is not auto-activated by hypoxia. Instead, EPOR seems to require a protein factor, but not EPO, which is produced by and secreted from hypoxic cancer cells.

Figure 5

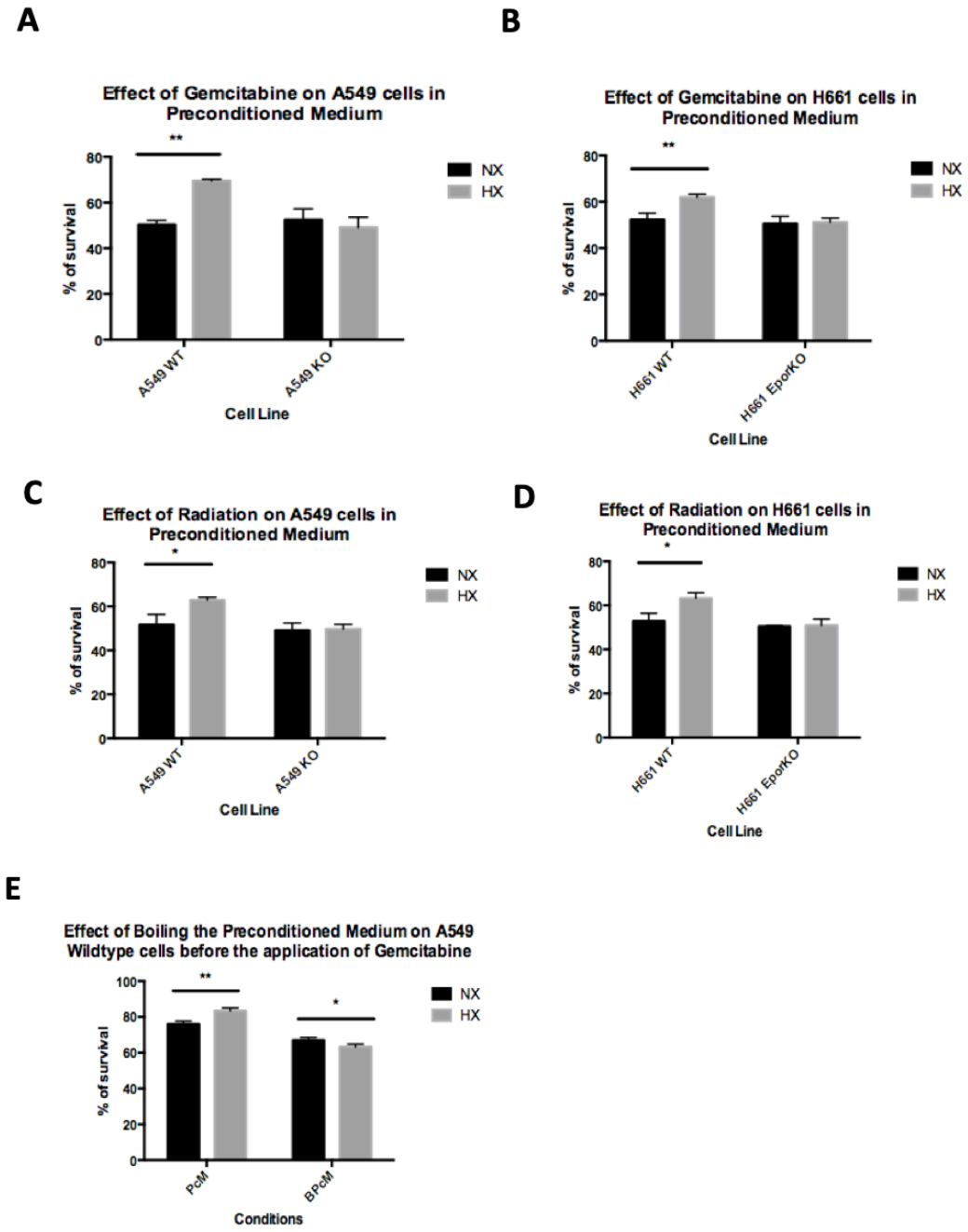


Fig. 5 Hypoxia-induced therapy resistance depends on EPOR

The survival rate of A549 and H661 lung cancer wildtype cells (WT) and EPO receptor knockout cells (EPORKO) was analyzed by clonogenic assay, after the cells were treated with Gemcitabine (A549 = 1.5ng/ml, H661= 2ng/ml), or radiation (3Gy). Beforehand they were kept in either normoxic (21% O_2) or hypoxic (1% O_2) preconditioned medium. The survival rate was analyzed by clonogenic assay and is shown in percent (%) to untreated controls of the respective EPOR expressing or deficient cell lines. Panels **A** and **B** show survival of in normoxic (21%, black), or hypoxic (1%, gray) preconditioned medium cultivated A549(**A**) (n=3) and H661 (**B**) (n=3) WT and EPORKO cells, in response to Gemcitabine treatment. Panels **C** and **D** show survival of in normoxic (21%, black), or hypoxic (1%, gray) preconditioned medium cultivated A549(**C**) (n=3) and H661 (**D**) (n=3) WT and EPORKO cells, in response to radiation treatment. Panel **E** shows in normoxic (21%, black) or hypoxic (1%, gray) preconditioned medium cultivated A549 WT cells (n=3) in response to Gemcitabine treatment.

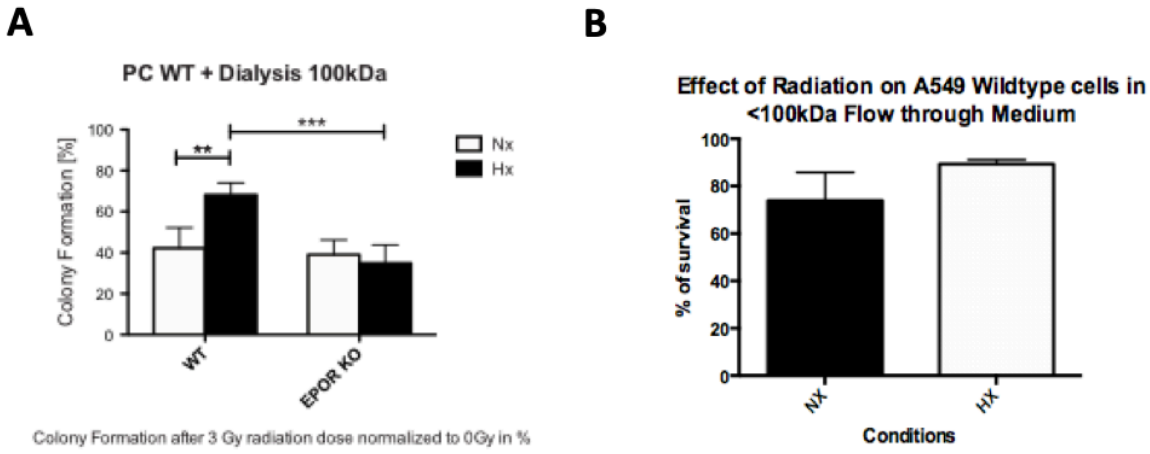
The preconditioned medium was either boiled before application (BPcM), or not (PcM). A Students t-test was performed for statistics. (Mean ± SD; n=3-4; *= $0.01 \leq p < 0.05$, **= $0.001 \leq p < 0.01$, *** = $p < 0.001$

5.5 Hypoxia-induced therapy resistance is mediated by Exosomes

To characterize the EPOR ligand that protects lung cancer cells from chemo- and radiotherapy, we harvested preconditioned medium and isolated protein fractions of different size. In a previous thesis, it was shown that the fraction with proteins larger than 100 kDa confers EPOR dependent protection of A549 lung cancer cells from radiation (Fig.6 A) [22]. Because most of the receptor ligands are smaller than 100 kDa [22], we hypothesized that exosomes, produced from hypoxia exposed cells, protect lung cancer cells from chemo- and radiotherapy. Exosomes are small vesicles that contain DNA, RNA, as well as proteins including receptor ligands [83]. The involvement of a receptor ligand transported by exosomes was supported by an experiment, where proteins larger than 100 kDa were removed from the hypoxia preconditioned medium. The medium, deprived of all molecules larger than 100 kDa, was still able to protect A549 lung cancer cells from radiation (Fig.6 B). This suggests that a normal receptor ligand is produced during hypoxia, which is both directly released into the medium as well as packed into exosomes. To test that assumption, we isolated exosomes from normoxia as well as hypoxia preconditioned medium and incubated A549 WT and EPORKO cells in it, prior to the Gemcitabine treatment. Our data show that exosomes isolated from hypoxia preconditioned medium increase the survival rate of Gemcitabine exposed A549 WT but not EPORKO cells from 72% to 87% ($p<0.05$) (Fig.7)

Our data suggests that the factor that protects lung cancer cells from chemo- and radiotherapy via EPOR is secreted under hypoxic conditions and is, at least partially, contained in an exosome.

Figure 6



[1]

Fig. 6 Radiation therapy resistance in A540 WT cells in medium <100 kDa
Panel **A** was taken with permission from the Dissertation of Nadine Jänicke [22]. Colony formation was analyzed via clonogenic assay after radiating the A549 WT and EPORKO cells with 3 Gy. Data was normalized to non-irradiated control in % (Y-axis). The preconditioned medium was preconditioned on WT cells for 24h in either normoxic (21% O_2; white bars) or hypoxic (1%O_2; black bars) conditions. This preconditioned medium (PC) was filtered by 100 kDa centrifugal filtration to remove all substances smaller than 100 kDa. WT and EPORKO cells were treated with the filtered, preconditioned medium 24h before the radiation treatment (3 Gy). Panel **B** shows the survival rate of A549 wildtype cells (WT) cultivated in normoxic (21%, black), or hypoxic (1%, gray) conditions, after radiotherapy with 3 Gy (n=2). The survival rate was analyzed by clonogenic assay and is shown in percent (%) to untreated controls of the respective EPOR expressing or deficient cell lines. A Students t-test was performed for statistics. (Mean ± SD; n=3-4; *= $0.01 \leq p < 0.05$, **= $0.001 \leq p < 0.01$, *** = $p < 0.001$

Figure 7

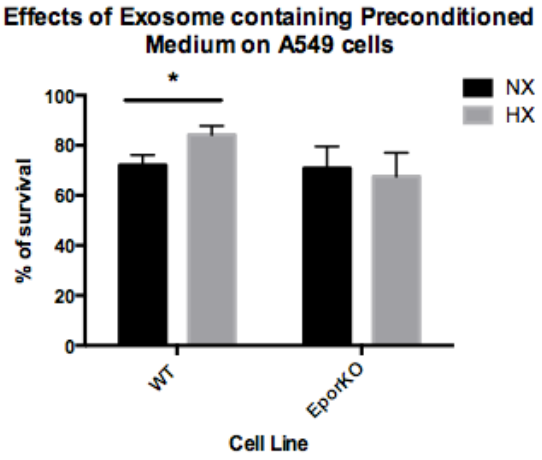


Fig. 7 Hypoxia-induced therapy resistance is mediated by Exosomes
The survival rate of A549 lung cancer wildtype cells (WT) and EPO receptor knockout cells (EPORKO) was

[1] Please note: Figure 6A was generated by Nadine Jänicke as part of her dissertation [22]

analyzed by clonogenic assay, after the application of extracted Exosomes, and radiotherapy (3 Gy) (n=3). Beforehand they were kept in either normoxic (21% O_2, black) or hypoxic (1% O_2, gray) preconditioned medium. The survival rate was analyzed by clonogenic assay and is shown in percent (%) to untreated controls of the respective EPOR expressing or deficient cell lines. A Students t-test was performed for statistics. (Mean ± SD; n=3-4; *= $0.01 \leq p < 0.05$, **= $0.001 \leq p < 0.01$, *** = $p < 0.001$

5.6 Alternative EPOR ligand candidates

To identify the factor that protects lung cancer cells via EPOR from chemo- and radiotherapy, we identified three potential candidates by literature research, which have been reported to be associated with EPO/EPOR activity: Stem cell factor (SCF) and Ephrin B2 (EFNB2) have been reported to be packed into extracellular vesicles [63]. SCF is a growth factor, which is required during EPO-induced maturation of erythroid progenitor cells during erythropoiesis [88] and either exists as a secreted or a membrane bound isoform, the latter potentially in the membrane of secreted vesicles [64]. The receptor of EFNB2 has previously been reported as being able to bind EPO [15] and thus, it might be possible that EFNB2 also binds EPOR. Furthermore, EPO seems to induce Thrombospondin-1 (THBS1) [89], the third potential candidate, whereas Thrombospondin-1 seems to be associated with liver metastasis and poor prognosis [65]. Thus, we tested if the *in silico* identified candidates SCF, EFNB2 and THBS1 protect A549 WT but not EPORKO cells from Gemcitabine. However, all three candidates neither protected A549 WT nor EPORKO cells from Gemcitabine (Fig.8).

Figure 8

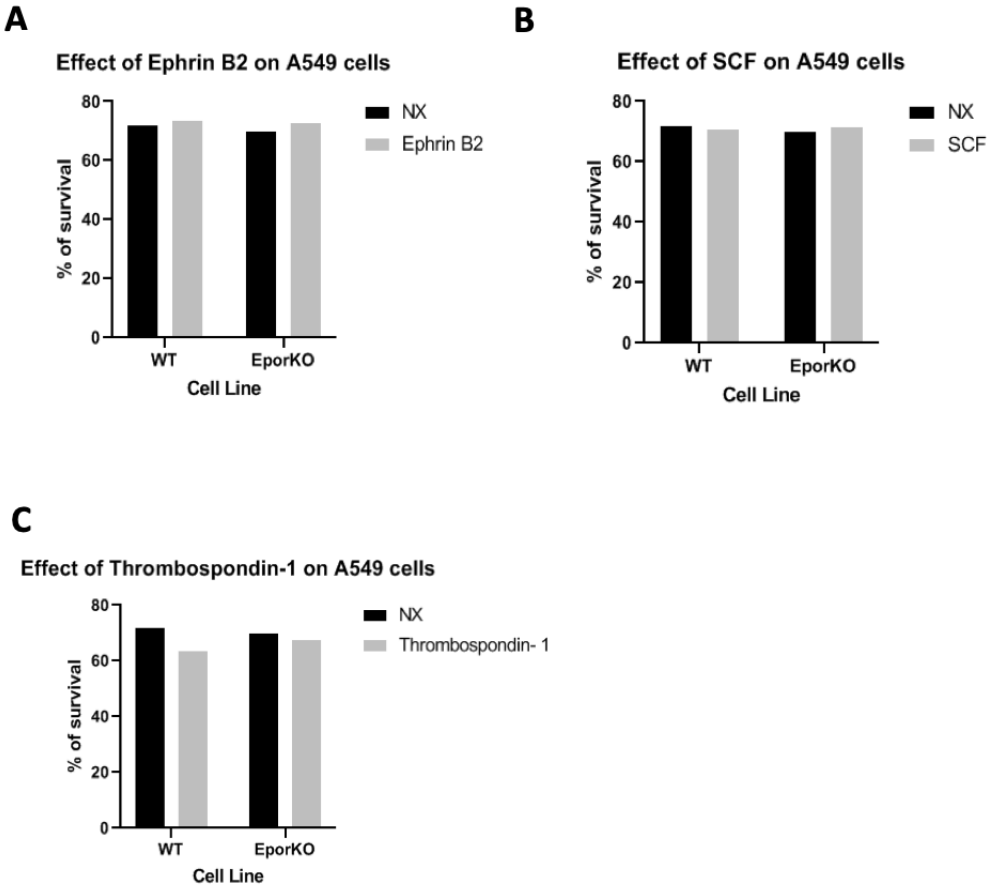


Fig 8: Application of the Growth Factors
The survival rate of A549 lung cancer wildtype cells (WT) and EPO receptor knockout cells (EPORKO) was analyzed by clonogenic assay, after the application of Ephrin B2, Stem Cell Factor, and Thrombospondin-1. Panel **A** shows survival of in normoxia (21 %) cultivated A549 WT and EPORKO in response to Gemcitabine treatment. Prior to treatment, either Ephrin B2 was applied (gray) or not (black) (n=1). Panel **B** shows survival of in normoxia (21 %) cultivated A549 WT and EPORKO in response to Gemcitabine treatment. Prior to treatment, either Stem Cell Factor was applied (gray) or not (black) (n=1). Panel **C** shows survival of in normoxia (21 %) cultivated A549 WT and EPORKO in response to Gemcitabine treatment. Prior to treatment, either Thrombospondin-1 was applied (gray) or not (black) (n=1). The survival rate was analyzed by clonogenic assay and is shown in percent (%) to untreated controls of the respective EPOR expressing or deficient cell lines. A Students t-test was performed for statistics. *= $0.01 \leq p < 0.05$, **= $0.001 \leq p < 0.01$, *** = $p < 0.001$

Because the three potential candidates did not protect A549 lung cancer cells, we analyzed the proteome of exosomes isolated from normoxic and hypoxic preconditioned medium of A549 cells by mass-spectroscopy. We used a qualitative, not a quantitative approach to identify the proteins that were contained in the exosomal fraction. The data shows that a large number of bovine proteins was identified, suggesting that the purification of the exosomal fractions was not entirely

clean. However, mass-spectroscopy is a very sensitive approach and small amounts of protein contamination can be easily identified [90]. Among the human proteins that were identified by mass-spectroscopy in the exosomal fraction, three candidates were detected that are potentially receptor binding ligands: 14-3-3ζ (YWHAZ) protein, Clusterin (CLU) and Inducible T- cell co- stimulator ligand (ICOSLG). Although the number of identified peptides of a protein is only a weak indicator of protein quantity, we detected more peptides in the exosomal fraction of hypoxic preconditioned medium, suggesting that these candidates might be upregulated by hypoxia. To test this, we quantified mRNA expression of normoxia and hypoxia exposed A549 lung cancer cells. Gene expression of both, ICOSLG (Fig.9 A) and YWHAZ (Fig.9 C) is induced 1.9 times and 2.2 times, respectively. CLU gene expression is not induced during hypoxia (Fig 9 B) and it might even be downregulated. Thus, we concluded that YWHAZ and ICOSLG are promising candidates, to be tested in future studies, that might confer protection against chemo- and radiotherapy in lung cancer cells in an EPOR dependent fashion.

Figure 9

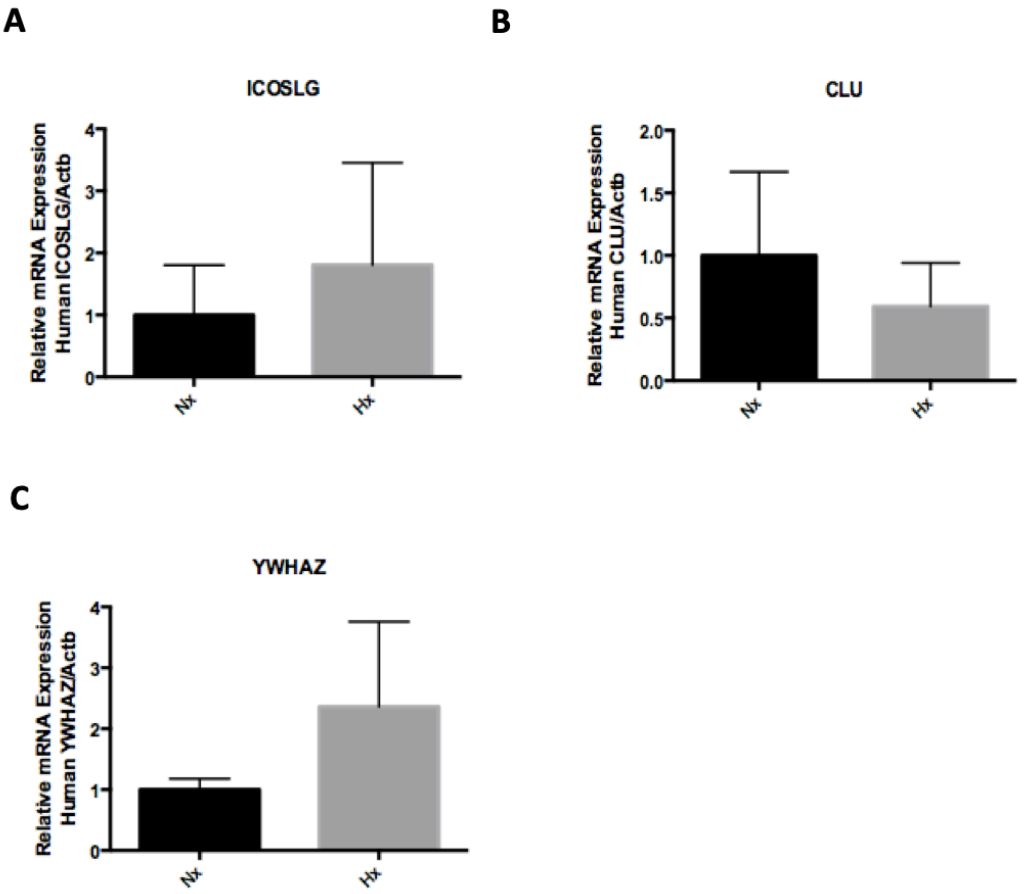


Fig. 9 Relative gene expression levels of ICOSLG, Clusterin and YWHAZ in normoxia and hypoxia exposed A549 cells.

Real Time PCR was performed using cDNA of the A549 WT in normoxic (21% O_2; black bars) and hypoxic (1% O_2; gray bars) conditions. The results obtained were normalized to β-actin (calibrator) and fold changes were calculated using the ΔΔCt method. The samples were run in duplicates. Panel **A** shows the *ICOSLG* expression of A549 WT cells (n=3). Panel **B** shows the *CLU* expression of A549 WT cells (n=3). Panel **C** shows the *YWHAZ* expression of A549 WT cells (n=3). A Students t-test was performed for statistics. (Mean ± SD; n=3-4; *= $0.01 \leq p < 0.05$, **= $0.001 \leq p < 0.01$, *** = $p < 0.001$

6. Discussion

In this study we illustrated the importance of EPOR in A549 and H661 lung cancer cells in maintaining therapy resistance. To test if EPO-EPOR protect lung cancer cells from chemo- and radiotherapy, we generated A549 and H661 EPOR knockout cells by CRISPR/Cas. We show in *in vitro* experiments that EPO neither protects lung cancer cells from chemo- nor from radiotherapy. However, the expression of EPOR is essential for hypoxia-induced protection of A549 and H661 lung cancer cell from chemo- and radiotherapy. We illustrate that the protective effect of EPOR under hypoxia is also EPO independent. Further, we display that hypoxia preconditioned medium protects EPOR expressing but not EPORKO lung cancer cells. This suggests that EPOR requires an alternative ligand, which is secreted by hypoxia-exposed cells, to protect cancer cells in a paracrine manner. Our data suggest that the ligand is a protein, because heat-inactivation reduces the protective effect of hypoxia preconditioned medium. Further, we show that this alternative EPOR ligand might be packed into and released from exosomes and we provide promising candidate ligands for future studies by analyzing the exosome proteome.

The large amount of bovine proteins in exosomes might result from cellular resorption of bovine proteins, which were packed into exosomes, although such a mechanism has not been described yet.

Cancer cells have been suspected to express EPOR [2], [13], [22] [59] and thus, EPO might increase tumor growth or malignancy. Indeed, a recent study showed EPOR protein expression in human cancer cell lines as well as human patients [16]. Preclinical studies showed that EPO and EPOR control proliferation of breast cancer stem cells [92], *in vivo* growth of glioma [92], as well as resistance to chemotherapy in a glioma mouse model [92]. However, the role of EPO/EPOR in protection of lung cancer cells from chemo- and radiotherapy has not yet been studied

We used two lung carcinoma cell lines, namely A549 and H661. A549 cells have previously been reported to express EPOR *in vitro* [22] and *in vivo* [93].We confirm EPOR expression of A549 cells *in vitro* and show that also H661 cells express EPOR *in vitro*. Afterwards, we generated EPOR knockout (EPORKO) clones by CRISPR/Cas to study the role of EPO/EPOR in lung cancer cell resistance against chemo- and radiotherapy.

Our data show that EPO administration does not protect A549 and H661 lung cancer cell from chemo- and radiotherapy. This contradicts the findings of Debeljak.N., et al, 2014 [112] who found a growth- promoting, anti-apoptotic action of EPO on MDA-MB-435 cells that were treated with radiation. Furthermore, Belenkov, et al, 2004 [57] showed that the presence of EPO in cultured media increases the survival of U87 and H100 cells after the application of cisplatin and radiation. However, although EPO did not protect A549 and H661 lung cancer cell from chemo- and radiotherapy, we show that the expression of EPOR is essential for better protection: Both, A549 and H661 cells are protected from radiation as well as Gemcitabine and Taxol when cultivated under hypoxic conditions. Hypoxia is a strong stimulus that activates cellular survival programs and protects healthy [95] and cancer cells [94], [95] from cytotoxic compounds. We show that the hypoxia-induced resistance to chemo- and radiotherapy is lost in EPOR lacking A549 and H661 cancer cells. Also, EPOR-lacking glioma cells have been reported to be more sensitive to Temozolomide *in vivo* [111], indicating that EPOR might play a critical role in cancer cell protection beyond lung cancer cells.

Why do lung cancer cells require EPOR for protection from chemo- and radiotherapy but do not respond to EPO treatment? In line with this study, a previously conducted study on EPOR expression in A549 lung cancer cells showed no measurable response to EPO [22]. Also, some breast cancer cells were reported to be non-responding to EPO despite a functional role of EPOR in cancer cells [96]. Thus, our data and the aforementioned studies suggest that EPOR might not require EPO to be activated. This independence of EPO could be explained by five different reasons, illustrated in Figure 10. Firstly, the expression of EPO could be induced during hypoxia, whereas the endogenous EPO of the cancer itself is increased. This could be enough to saturate all the EPOR, resulting in no further EPOR activation by the application of rhEPO [97]. Secondly, EPOR might require a co- stimulating factor, which could be another reason for why the cancer cells did not respond to rhEPO. This theory states that while EPO binds to EPOR, another ligand, Factor X, needs to bind to its corresponding Receptor X, for therapy-resistance to occur [22]. Therefore, solely applying rhEPO did not allow for the desired effect to occur. Another possibility is that although the FCS used was heat-inactivated, the bovine EPO is still (partially) active and capable of binding the human EPOR, as suggested by Belenkov, et al, 2004

[57]. In this scenario, all the EPORs on cancer cells were already saturated and fully activated by bovine EPO before the application of rhEPO. As a fourth reason, non-ligand induced activation could account for the non-responsiveness of EPOR to the administered rhEPO. As shown by Kourounioutis, et al, 2016 [82], certain receptors are activated without the binding of a ligand. Lastly, an alternative ligand [22] could bind to EPOR, making us see no effect when applying rhEPO. EPO can act as an alternative ligand for the Ephrin B4 receptor [15]. It is not clear whether EphrinB2 (EFNB2), or another alternative ligand, may also be able to bind and activate the heterodimeric isoform of EPOR, which is the isoform where EPO has a reduced affinity to.

Figure 10

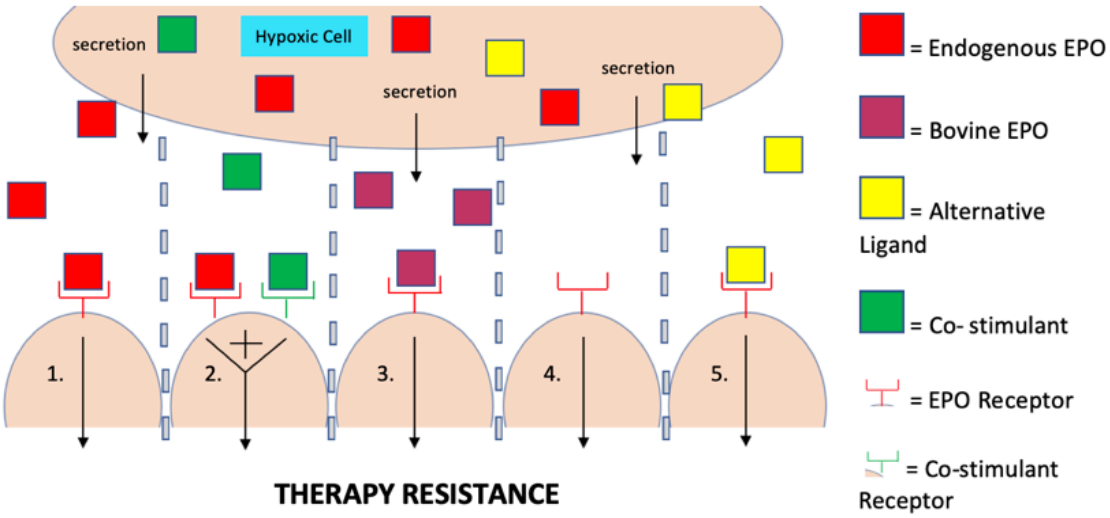


Fig.10 Possible scenarios of how EPOR induces therapy resistance in hypoxia
Illustrated are the five possible scenarios of EPO-dependent and independent mechanisms that result in resistance. Scenario 1 illustrates endogenously produced EPO by cancer cells, which binds to EPOR. Scenario 2 suggests a co-stimulation, where EPO binds to the EPOR but simultaneously requires a co-stimulus to protect cancer cells. In scenario 3, bovine not human EPO (contained in FCS) binds to EPOR. Scenario 4 shows the non-ligand induced auto-stimulation of EPOR and scenario 5 illustrates the alternative ligand theory, where an alternative ligand instead of EPO binds to EPOR.

To decipher how EPOR protects lung cancer cells from chemo-and radiotherapy during hypoxia, we tested if endogenously produced EPO during hypoxia activates EPOR. However, when we used soluble EPOR prior to radiation to sequester endogenous EPO potentially secreted by the cancer cells, the hypoxic lung cancer

cells were still protected from chemo- and radiotherapy. Thus, neither endogenously produced nor FCS-born bovine EPO are required for EPOR activation in hypoxic lung cancer cells. To confirm these results in an independent experiment, we used the EPOR antagonist EMP9 [81]. The incubation of EMP9 did not prevent EPOR dependent protection of hypoxia-exposed cells from chemo- and radiotherapy. This experiment confirms that EPO is not required for EPOR dependent protection. Furthermore, it implies that EPOR may not exist in a homodimeric form in lung cancer cells. The existence of EPOR as a heterodimer has been proposed [58] and verified [23]. We detected the expression of the b-common receptor (CD131) in A549 lung cancer cells (supplemental Fig. 4), suggesting that the heterodimeric EPOR might exist in lung cancer cells. However, also the EPOR heterodimer with the β-common receptor is EPO responsive [98], suggesting that there might be a third EPOR version in cancer cells, by which the hypoxia-induced therapy resistance functions. Thus, we concluded that EPOR dependent protection from chemo- and radiotherapy is completely independent of EPO. Neither endogenously expressed or administered human nor FCS-born bovine EPO as a single stimulant or as a co-stimulant with a second factor, are involved in hypoxia-induced cell protection.

To test the two remaining scenarios, the non-ligand induced autoactivation of EPOR as described by Naranda, et al, 2002 [113], and the alternative ligand scenario, we used hypoxia preconditioned medium to determine if a secreted factor is enough to protect lung cancer cells from chemo- and radio- resistance.

In fact, hypoxia preconditioned medium was enough to protect normoxia incubated A549 and H661 WT but not EPORKO cells from chemo- and radiotherapy. This suggest that EPOR requires a ligand and might not be activated in a non-ligand induced fashion. When the hypoxia preconditioned medium was heat-inactivated, normoxic cells were not protected, which suggest that the ligand is protein-based. In previous experiments we showed that the ligand is bigger than 100 kDa [22]. Due to the size, we hypothesized that the ligand is packed into exosomes, which are produced by cancer cells [99] and can increase tumor malignancy and resistance against therapeutic interventions [100]. Indeed, we were able to show that an exosome fraction, isolated from preconditioned medium, protects normoxia-incubated WT but not EPORKO cells. However, also the medium fraction containing all molecules smaller than 100 kDa had a protective effect, suggesting, that the ligand exists as both,

exosome-bound as well as free form. To identify the ligand, we analyzed the exosome isolated proteome from normoxic and hypoxic preconditioned medium. We identified 83 proteins, which is a little less than cited in the previous literature [101]. Among the identified proteins, we discovered three proteins that are able to act as ligands: 1. YWHAZ, is a protein that classically binds to a large spectrum of partners such as; cadherine binding, identical protein binding, ion channel binding, RNA binding, etc. [103]. It is frequently upregulated in various types of cancers, acting as an oncogene by increasing cell growth, cell cycle, apoptosis, migration, invasion and metastasis [60]. Further, YWHAZ interacts with HIF-1α under hypoxic conditions and enhances its protein stability, inducing cell migration and invasion. Recent studies showed that Gemcitabine resistant cells upregulated the expression of YWHAZ [44], suggesting that it is a promising candidate to evaluate. 2. We identified Clusterin that suppresses DNA damage-induced cell death in tumor cells, and is often overexpressed in breast-cancer [45]. Clusterin binds to the endocytic receptor Megalin [105] and was thus, considered to be a potential ligand [104]. 3. The third identified protein was ICOSLG, which has been reported to be associated with a poor prognosis in patients with Glioblastoma [51]. ICOSLG induces IL-10 production in T-cells, which further promotes regulatory T-cell- mediated peripheral tolerance [51]. T- Lymphocytes can be separated into three different groups based on their function; Cytotoxic T Cells (CD 8+), Helper Cells (CD 4+), and regulatory T Cells (Tregs) [106]. Tregs play an essential role in maintaining self-tolerance. Therefore, they are capable of suppressing anticancer immunity, thus promoting tumor development and progression[107]. Through its influence on Tregs, ICOSLG was another candidate to look into.

We analyzed if the gene expression of the three candidate proteins was hypoxia-inducible to estimate if the three candidates are involved in EPOR-mediated protection of A549 and H661 lung cancer cells from chemo- and radiotherapy. We observed that gene expression of both, YWHAZ and the ICOSLG was slightly increased during hypoxia. In contrast, Clusterin was expressed at an even lower rate during hypoxia. Thus, YWHAZ and ICOSLG are two promising candidates that might stimulate an EPOR-heterodimer and protect lung cancer cells from chemo- and radiotherapy, as illustrated in Figure 11.

Figure 11

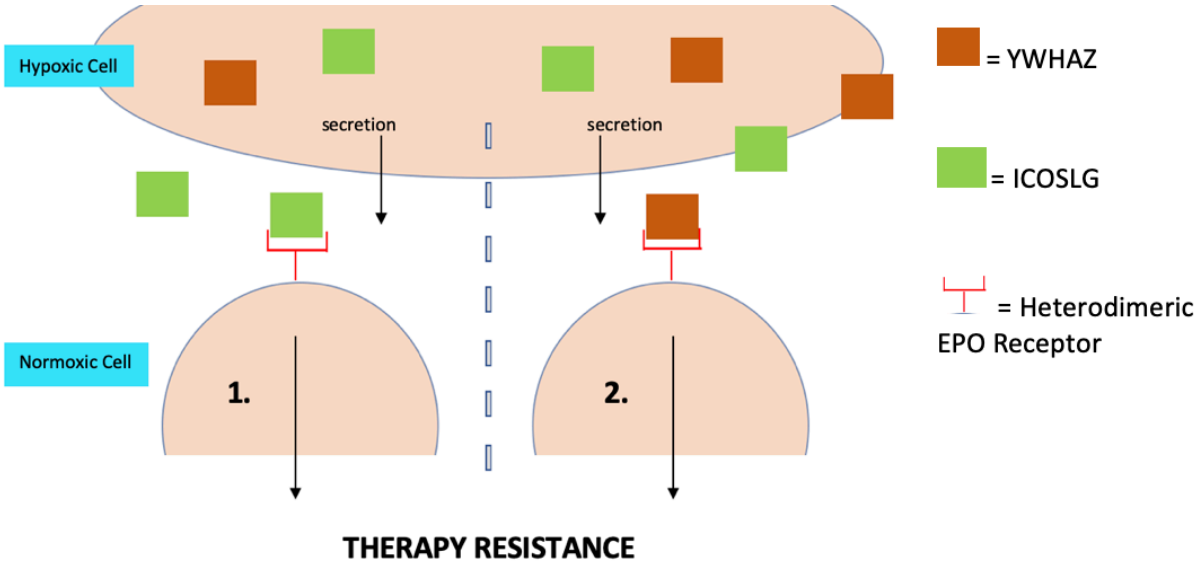


Fig.11 A new hypothesis: Alternative ligands stimulate a heterodimeric EPO Receptor
Illustrated are two possible ligands, YWHAZ and ICOSLG, which were found in exosomes and could stimulate the, potentially heterodimeric, EPOR resulting in hypoxia- induced chemo- and radio-resistance. Scenario 1 illustrates how ICOSLG could function as a possible alternative ligand binding on a heterodimeric EPO Receptor. Scenario 2 displays another possible ligand, YWHAZ, binding on to a heterodimeric EPO Receptor, which leads to the hypoxia induced therapy resistance.

6.1 Outlook

The future goal is to identify the ligand that binds a heterodimeric form of EPOR resulting in therapy resistance of A549 and H661 lung cancer cells. Because YWHAZ and ICOSLG were identified in exosomes of hypoxia preconditioned medium, and both promote cancer, both factors are promising candidates for future research projects. Both proteins could be applied to or overexpressed in A549 and H661 WT cells under normoxic conditions, to test their potential to protect lung cancer cells from chemo- and radiotherapy. EPORKO cells can be used to test if the ligands, given that they mediate cancer cell protection, protect by binding or interacting with EPOR. An aspect that has not been addressed in our study is the classical EPOR/JAK2/STAT pathway: In a future study, it is important to analyze if therapy resistance is mediated via the classical EPOR/JAK2/STAT pathway, which can be tested by AG490, a JAK2 inhibitor to specifically target the JAK2/STAT pathway [22], [61], [62].

6.2 Conclusion

In conclusion, we showed that EPOR is required for hypoxia-induced resistance to chemo- and radiotherapy in A549 and H661 lung cancer cells. We further show that EPO is not required for the EPOR-mediated protection and suggest an alternative ligand that binds a (potentially) heterodimeric EPO Receptor. Although we show that EPOR but not EPO itself protected lung cancer cells from therapeutic approaches, we recommend that EPO treatment of anemic cancer patients is handled with care. However, our study indicates that cancer-specific targeting of the EPOR could be a possible strategy to increase the sensitivity of cancers cells to chemo- and radiotherapy, especially in hypoxic tumor regions. For that, the identification of the EPOR binding ligand as well as the identification of the EPOR heterodimer is essential and the next step to take.

7. References

1. Jelkman, W., *Physiology and Pharmacology of Erythropoietin*. Transfus Med Hemother, 2013. **40**(5): p. 302-309
2. Jelkmann, W., et al., *The erythropoietin receptor in normal and cancer tissues*. Crit Rev Oncol Hematol, 2008. **67**(1): p. 39-61.
3. Karen, S., et al., *EPO does not promote interaction between the erythropoietin and beta – common receptors*. Scientific Reports, 2018. **8** (12457)
4. Brines, M., et al., *Erythropoietin mediates tissue protection through an erythropoietin and common beta-subunit heteroreceptor.* Proc Natl Acad Sci U S A, 2004.**101**(41): p.14907-14912.
5. Masuda,S., et al., *Functional Erythropoietin Receptor of the Cells With Neural Characteristics. Comparison with Receptor Properties of Erythroid Cells.* The Journal of Biological Chemistry, 1993. **268**(15): p. 11208-11216.
6. Pinevich. AJ., et al., *Erythropoietin therapy in patients with chronic renal failure.* West J Med, 1992. **157**(2): p.154-157
7. Temkin, S.M., et al., *Erythropoietin administration during primary treatment for locally advanced cervical carcinoma is associated with poor response to radiation*. Int J Gynecol Cancer, 2006. **16**(5): p. 1855-61
8. Caro, J.J., et al., Anemia as an independent prognostic factor for survival in patients with cancer: a systemic, quantitative review. Cancer, 2001. **91**(12): p. 2214-21.
9. Belda-Iniesta., C., et al., *Human recombinant erythropoietin does not promote cancer growth in presence of functional receptors expressed in cancer cells.* Cancer Biology& Therapy, 2007.
10. Hardee, ME., et al., *Human recombinant erythropoietin (rEpo) has no effect on tumour growth or angiogenesis.* Br J Cancer, 2005. **93**: p. 1350–1355
11. Fandrey, J., *Erythropoietin receptors on tumor cells: what do they mean?* Oncologist, 2008. **3**: p. 16-20.
12. Pinevich. AJ., et al., *Erythropoietin therapy in patients with chronic renal failure.* West J Med, 1992. **157**(2): p.154-157
13. Acs, G., et al., *Erythropoietin and erythropoietin receptor expression in human cancer*. Cancer Res, 2001. **61**(9): p. 3561-5.
14. Arcasoy, MO., et al., *Erythropoietin and erythropoietin receptor expression in human prostate cancer.* Modern Pathology, 2004. **18**: p. 421-430
15. Pradeep, S., et al., *Erythropoietin Stimulates Tumor Growth via EphB4*. Cancer Cell, 2015. **28**(5): p. 610-22.
16. Maxwell, P., et al., *Novel antibodies directed against the human erythropoietin receptor: creating a basis for clinical implementation*. British journal of Haematology, 2015. **168**(3): p. 429-442
17. Jing, X., et al., *Role of Hypoxia in cancer therapy by regulating the tumor microenvironment.* Molecular Cancer, 2019. **18** (157)
18. Harrison, L., et al., *Hypoxia and anemia: factors in decreased sensitivity to radiation therapy and chemotherapy?.* Oncologist, 2004. **9**(5): p. 31-40
19. Dagnon. K., et al., *Expression of erythropoietin and erythropoietin receptor in non-small cell lung carcinomas.* Clin Cancer Res, 2005. **11**: p. 993–999
20. Peres, E.A., et al., *Silencing erythropoietin receptor on glioma cells reinforces efficacy of temozolomide and X-rays through senescence and mitotic catastrophe*. Oncotarget, 2015. **6**(4): p. 2101-19.
21. Thornton, B., et al., *Real-Time PCR (qPCR) Primer Design using free online Software*. Biochemistry and Molecular Biology Education, 2011. **39** (2):p. 145-154.

22. Jänicke, Nadine: Hyoxia-induced Resistance to Radiotherapy in Lung Cancer Cells is mediated by the Erythropoietin Receptor.,unv.Diss.,Universität Zürich, 20<.
23. Cheung Tung Shing, K.S., et al., *EPO does not promote interaction between the erythropoietin and beta-common receptors*. Scientific Reports, 2018. **8** (1):p.12457
24. Frille, A., et al., *No erythropoietin- induced growth is observed in non-small cell lung cancercells*. International Journal of Oncology, 2017. **52** (2):p.518-526
25. Miao,S., et al., *Erythropoietin promoted the proliferation of hepatocellular carcinoma through hypoxia induced translocation of its specific receptor*. Cancer Cell International, 2017. **17:** p. 119
26. Orsini, M., et al., *Sphingolipid-mediated inflammatory signaling leading to autophagy inhibition converts erythropoiesis to myelopoiesis in human hematopoietic stem/progenitor cells*. Cell Death &Differentiation, 2018. doi: 10.1038/s41418-018-0245-x
27. Wang, P., et al., *AMP-activated kinase- dependent induction of autophagy by erthyropoietin protects against spinal cord injury in rats*. CNS Neuroscience & Therapeutics , 2018. **24** (12): p. 1185-1195
28. Nishimura,K.,et al., *Effect of Erythropoietin production induced by hypoxia on autophagy in HepG2 cells*. ScienceDirect, 2017. **495** (1): p.1317-1321
29. Rusten, E.T., et al., *P62, an autophagy hero or culprit?*. 2010. **12**: p. 207-209
30. Kittur, F.S., et al., *Recombinant asialoerythropoietin protects HL-1 cardiomyocytes from injury via suppression of Mst1 activation*. 2019. **17:** p.157-168
31. Miao, S., et al., *Erythropoietin promoted the proliferation of hepatocellular carcinoma through hypoxia induced translocation of its specific receptor,* Cancer Cell International, 2017. **17**
32. Arcasoy, M., et al., *Expression of erythropoietin recpetor splice variants in human cancer*. ScienceDirect, 2003.**307** (4): p.999-1007
33. Labs, New England Bio. *New England BioLabsInc.* . 2019. http://international.neb.com/protocols/0001/01/01/high-efficiency-transformation-protocol-c2987 (accessed 09 20, 2019).
34. *Basic Local Alignement Search Tool*. http://blast.ncbi.nlm.nhi.gov/Blast.cgi?PAGE_TYPE=BlastSearch&BLAST_SPEC=blast2seq&LINK_LOC=align2seq (accessed 03 15, 2019).
35. *ExPASy*. http://web.expasy.org/translate/ (accessed 3 15, 2019).
36. Fayazi,M., et al., *The effect of stem cell factor on proliferation of human endometrial CD146 cells*. Int J Reprod BioMed, 2016. **14**(7): p.437-442
37. Maekawa, H.,et al., *Ephrin-B2 Induces migration of Endothelial Cells Through the Phosphatidylinositol-3 Kinase Pathway and Promotes Angiogenesis in Adult Vasculature*. Arteriosclerosis, Thrombosis, and Vascular Biology, 2003. **23**: p.2008-2014
38. Yoshida, J., et al., *Intervenolin suppresses gastric cancer cell growth through the induction of TSP-1 secretion from fibroblast-like stromal cells*. Spandidos Publications, 2018.: p.6777-6785
39. Koressaar, T.,et al., *Enhancements and modifications of primer design program Primer3*. Bioinformatics, 2007. **23**(10): p. 1289-91.
40. Untergasser, A., et al., *Primer3--new capabilities and interfaces*. Nucleic Acids Res, 2012. **40**(15): p. e115.
41. Liu, W.M., et al., *Effect of haemopoietic growth factors on cancer cell lines and their role in chemosensitivity*. Oncogene, 2004. **23**(4): p. 981-90.
42. Laugsch, M., et al., *Lack of functional erythropoietin receptors of cancer cell lines*. Int J Cancer, 2008. **122**(5): p. 1005-11.

43. Lei,H., et al., *Local blockage of self-sustainable erythropoietin signaling suppresses tumor progression in non-small cell lung cancer*. Oncotarget, 2017.**8**(47): p. 82352-82365
44. Qiu, YJ., et al., *14-3-3 Protein mediates gemcitabine resistance in NK/T-cell lymphoma*. Zhonghua Xue Ye Xue Za Zhi, 2019. **40** (11): p. 906-911
45. Ming, X., et al., *Clusterin, a novel DEC1 Target, Modulates DNA Damage-Mediated Cell Death*. Mol Cancer Res, 2018. **16** (11): p. 1641-1651
46. Metzen, E., et al., *Regulation of the prolyl hydrocylase domain protein 2 (phd2legln-1) gene: identification of a functional hypoxia-responsive element*. Biochemical Journal, 2005. **387** (Pt 3): p. 711-717
47. Peres, E.A., et al., *Silencing erythropoietin receptor on glioma cells reinforces efficacy of temozolomide and X-rays through senescence and mitotic catastrophe*. Oncotarget, 2015. **6**(4): p. 2101-19.
48. Bribes, M., et al., *The Receptor that tames the innate Immune response*. Literature Review, 2011. **18**(1): p.486-496
49. Yu, CC., et al., *Genetic Association Analysis of Cell Cycle Regulators Reveals YWHAZ Has Prognostic Significance in Prostate Cancer*. Cancer Genomics Proteomics, 2020. **17**(2): p. 209-216
50. Yang, B., et al., *MiRNA- 802 suppresses proliferation and migration of epithelial ovarian cancer cells by targeting YWHAZ*. J Ovarian Res, 2019. **12**(1)
51. Iwata, R., et al., *ICOSLG- mediated regulatory T cell expansion and IL-10 production promote progression of glioblastoma*. Neuro Oncol, 2019. doi: 10.1093/neuonc/noz204.
52. Levine, B., et al., *Biological Functions of Autophagy Genes: A Disease Persepective*. Cell, 2020. **176**(1-2): p.11- 42
53. Amaravadi, R., et al., *Recent insights into the function of autophagy in cancer*. Genes & Development, 2016. **1;30**(17): p.1913-1930
54. Degenhardt, K., et al., *Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis*. Cancer Cell, 2006. **10**: p.51–64
55. Chapman, J.D., et al., *Measuring hypoxia and predicting tumor radioresistance with nuclear medicine assays*. Radiother Oncol, 1998. **46**(3): p. 229-37
56. Murray, D., et al., *Influence of oxygen on the radiosensitivity of human glioma cell lines*. Am J Clin Oncol, 2003. **26**(5): p. e169-77.
57. Belenkov, A.I., et al., *Erythropoietin induces cancer cell resistance to ionizing radiation and to cisplatin*. Mol Cancer Ther, 2004. **3**(12): p. 1525-32.
58. Watowich, S.,et al., *The Erythropoietin Receptor: Molecular Structure and Hematopoietic Signaling Pathways*. J Investig Med, 2011. **59**(7): p. 1067-1072
59. Dunlop, E.A, et al., *Induction of Signalling in Non- Erythroid Cells by Pharmacological Levels of Erythropoietin*. Neurodegenerative Diseases, 2006. **3**: p.94.100
60. Gan, Y., et al., *The role of YWHAZ in cancer: A maze of opportunities and challenges*. J Cancer, 2020. **11**(8): p.2252-2264
61. Samanta, A.K., et al., *Janus kinase 2: a critical target in chronic myelogenous leukemia*. Cancer Res, 2006. **66**(13): p. 6468-72.
62. Miyamoto, N., et al., *The JAK2 inhibitor AG490 predominantly abrogates the growth of human B-precursor leukemic cells with 11q23 translocation or Philadelphia chromosome*. Leukemia, 2001. **15**(11): p. 1758-68.
63. Quesenberry, P.J., et al., *Role of extracellular RNA-carrying vesicles in cell differentiation and reprogramming*. Stem Cell Res Ther, 2015. **6**: p. 153.

64. Slanicka Krieger, M., et al., *The membrane-bound isoform of stem cell factor synergizes with soluble flt3 ligand in supporting early hematopoietic cells in long-term cultures of normal and aplastic anemia bone marrow*. Exp Hematol, 1998. **26**(5): p. 365-73.
65. Liu, X., et al., *THBS1 facilitates colorectal liver metastasis through enhancing epithelial- mesenchymal transition*. Clin Transl Oncol, 2020. doi: 10.1007/s12094-020-02308-8
66. Broxmeyer, HE., et al., *Erythropoietin: multiple targets, actions, and modifying influences for biological and clinical consideration*. J Exp Med, 2013. **210** (2): p. 205- 208
67. Frille, A., et al., *No erythropoietin- induced growth is observed in non-small cell lung cancer cells*. International J Oncol, 2018. **52**(2): p. 518-526
68. Swift, S., et al., *Absence of functional EpoR expression in human tumor cell lines*. Blood, 2010. **115**(21): p. 4254-63.
69. https://www.cancer.org/cancer/non-small-cell-lung-cancer/about/what-is-non-small-cell-lung-cancer.html.
70. Westenfelder, C., et al., *Erythropoietin stimulates proliferation of human renal carcinoma cells*. Kidney Int, 2000. **58**: p. 647–657
71. Feldman. L., et al., *Erythropoietin stimulates growth and STAT5 phosphorylation in human prostate epithelial and prostate cancer cells,* 2006. Prostate. **66**: p. 135–145.
72. Peres, E.A., et al., *Targeting the erythropoietin receptor on glioma cells reduces tumour growth*. Exp Cell Res, 2011. **317**(16): p. 2321-32.
73. Bohlius. J., et al., *Recombinant human erythropoiesis-stimulating agents and mortality in patients with cancer: A meta-analysis of randomised trials.* Lancet, 2009. **373**: p. 1532–1542
74. Denlinger, C.E., B.K. Rundall, and D.R. Jones, *Proteasome inhibition sensitizes non-small cell lung cancer to histone deacetylase inhibitor-induced apoptosis through the generation of reactive oxygen species*. J Thorac Cardiovasc Surg, 2004. **128**(5): p. 740-8.
75. cancer.org, https://www.cancer.org/cancer/non-small-cell-lung-cancer/about/what-is-non-small-cell-lung-cancer.html.
76. Mahmood,T., et al., *Western blot: technique, theory, and trouble shooting*. North American journal of medical sciences, 2012. **4**(9): p. 429- 434.
77. Shuo Miao., et al., *Erythropoietin promoted the proliferation of hepatocellular carcinoma through hypoxia induced translocation of its specific receptor*. Cancer Cell International, 2017. **17**: p. 119
78. Yugi, L., et al., *Nonerythropoetic Erythropoietin- Derived Peptide suppresses Adipogenesis, Inflammation, Obesity and Insulin Resistance*. Scientific Reports, 2015. **5** (15134)
79. Viraj P, M., et al., *Systematic Method for determining an Ideal Housekeeping Gene for Real-Time PCR Analysis*. J Biomol Tech, 2008. **19**(5): p.342-347
80. Hayashi, H., et al., *Gemcitabine: Efficacy in the Treatment of Advanced Stage Nonsquamous Non- Small Cell Lung Cancer*. Clinical Medicine Insights: Oncology, 2011. **5**: p. 177-184
81. Yasuda, Y., et al., *Significance of Erythropoietin Receptor Antagonist EMP9 in Cancers*. Vitam Horm, 2017. **105**: p. 297-310
82. Kourounioutis, G., et al., *Non- Ligand-Induced Dimerization is Sufficient to Initiate the Signalling and Endocytosis of EGF Receptor*. Int J Mol Sci, 2016. **17**(8)

83. Raghu Kalluri., *The biology and function of exosomes in cancer*. J Clin Invest, 2016. **126** (4): p. 1208-1215
84. Borrego-Soto, G., et al., *Ionizing radiation-induced DNA injury and damage detection in patients with breast cancer*. Genet Mol Biol, 2015. **38**(4): p. 420-432
85. Bristow, R.G. and R.P. Hill, *Hypoxia and metabolism. Hypoxia, DNA repair and genetic instability*. Nat Rev Cancer, 2008. **8**(3): p. 180-92.
86. Gewirtz, D.A., *The Autophagic Response to Radiation: Relevance for Radiation Sensitization in Cancer Therapy*. Radiation Research, 2014. **182** (4): p.363-367
87. Maiese, K., *Regeneration in the nervous system with erythropoietin*. Front Biosci (Landmark Ed), 2016. **21**(3): p. 561-596
88. Sato,T., et al., *Erythroid progenitors differentiate and mature in response to endogenous Erythropoietin*. J Clin Invest, 2000. **106**(2): p263-270
89. Gillinder, K., et al., *Direct targets of pSTAT5 signalling in erythropoiesis*. PLOS ONE, 2017.
90. Rardin, M.J., *Rapid Assessment of Contaminants and Interferences in Mass Spectrometry Data Using Skyline*. Journal of The American Society for Mass Spectrometry, 2018.p: 1327-1330
91. Sytkowski, A.J., *Does erythropoietin have a dark side? Epo signaling and cancer cells*. Sci STKE, 2007. **2007**(395): p. pe38.
92. Pérès. E., et al., *Targeting the erythropoietin receptor on glioma cells reduces tumour growth.*Exp Cell Research, 2011. **317**(16): p.2321-2332.
93. Fuge. F., et al., *In-vivo detection of the erythropoietin receptor in tumours using positron emission tomography.* Molecular Imaging, 2014.
94. Eales. K.L., et al., *Hypoxia and metabolic adaptation of cancer cells.* Oncogenesis, 2016. **5**
95. Jing. X., et al., *Role of hypoxia in cancer therapy by regulating the tumor microenvironment.* Molecular Cancer, 2019.**18** (157)
96. Reinbothe. S., et al., *EPO-independent functional EPO receptorin breast cancer enhances estrogen receptor activity and promotes cell proliferation.* Biochem Biophys Res Commun, 2014. **445**(1): p.163-169
97. Miao. S., et al., *Erythropoietin promoted the proliferation of hepatocellular carcinoma through hypoxia induced translocation of its specific receptor.* Cancer Cell International, 2017. **119**
98. Broxmeyer. H., *Erythropoietin: multiple target, actions, and modifying influences for biological and clinical consideration.* Journal of Experimental Medicine, 2013. **210** (2): p.205-208
99. Skog. J., et al., *Glioblastoma micorvesicles transport RNA and protein that promote tumor growth and provide diagnostic biomarkers.* Nat Cell Biol, 2008. **10** (12): p.1470-1476
100. Azmi. A., et al., *Exosomes in Cancer Development, Metastasis and Drug Resistance: A Comprehensive Review.* Cancer Metastasis Review, 2013. **32**(0)
101. Smolary. M., et al., *Proteome Profiling of Exosomes Purified from a Small Amount of Human Serum: The Problem of Co-Purified Serum Components.* Proteomes, 2019
102. Popov. I., et al., *A YWHAZ Variant Associated With Cardiofaciocutaenous Syndrome Activates the RAF- ERK Pathway.*Frontiers in Physiology, 2019
103. https://www.uniprot.org/uniprot/P63104
104. Peng. M., et al., *The Role of Clusterinin cancer metastasis.* Cancer Manag Res, 2019. **11**: p2405-2414

105. Chaiwatanasirikul. K-A., *The Tumour- suppressive function of CLU is explained by its localization and interaction with HSP60.* Cell Death & Disease, 2011
106. https://www.sinobiological.com/research/cd-antigens/cluster-of-differentiation-t-lymphocyte
107. Togashi. Y., et al., *Regulatory T cells in cancer immunosuppression-implications for anticancer therapy.* Nature Reviews Clinical Oncology, 2019. **16**: p. 356-371
108. Shiozawa. Y., et al., *Erythropoietin Supports the Survival of Prostate Cancer, But not Growth and Bone Metastasis.* J Cell Biochem, 2013.**114**(11): p.2471-2478
109. Thuc-Nghi Duc Pham., et al., *Erythropoietin inhibits chemotherapy induced cell death and promotes a senescence- like state in leukemia cells.*Cell Death and Disease, 2019. **10**(22)
110. Trošt, N., et al., *Correlation between erythropoietin receptor(s) and estrogen and progesterone receptor expression in different breast cancer cell lines.* Int J Mol Med, 2013. **31**(3): p. 717–25.
111. Pérès. E., et al., *Silencing erythropoietin receptor on glioma cells reinforces efficacy of temozolomide and X-rays through senescence and mitotic catastrophe.* Oncotarget, 2014. **6**(4)
112. Debeljak. N., et al., *Erythropoietin and Cancer: The Unintended Consequences of Anemia Correction.* Frontiers in Immunology, 2014. **5**: p. 563
113. Naranda. T., et al., *Activation of Erythropoietin Receptor through a Novel Extracellular Binding Site.* Endocrinology, 2002. **143** (6): p. 2293-2302
114. Saintigny, P., et al., *Erythropoietin and erythropoietin receptor coexpression is associated with poor survival in stage I non-small cell lung cancer*. Clin Cancer Res, 2007. **13**(16): p. 4825-31.
115. Jeong, J.Y., et al., *Characterization of erythropoietin receptor and erythropoietin expression and function in human ovarian cancer cells*. Int J Cancer, 2008. **122**(2): p. 274-80.
116. Swift, S., et al., *Absence of functional EpoR expression in human tumor cell lines*. Blood, 2010. **115**(21): p. 4254-63.
117. Ostrowski, D., et al., *Alternative Erythropoietin Receptors in the Nervous System*. Journal of clinical medicine, 2018. **7** (2)
118. Arcasoy, MO., et al., *Functional significance of erythropoietin receptor expression in breast cancer.* Lab Invest, 2002. **82**(7): 911-918.
119. Sander, J.D., et al., *CRISPR-Cas systems for genome editing, regulation and targeting*. Nature Biotechnoliogy, 2014. **32**(4): p.347-355
120. Brown, JM., *Tumor Hypoxia in Cancer Therapy.* Methods Enzymol, 2007. **435**: p. 297-321
121. Kaiwen, I.L., et al., *Rapid control of Genome Editing in Human Cells by Chemical-Inducible CRISPR-Cas Systems*. Synthetic Biology, 2018. Vol.1772: p. 267-288
122. Cantarelli, C., et al., *Erythropoietin, a multifaced protein with innate and adaptive immune modulatory activity.* American Journal of Transplantation, 2019.
123. Lei, H., et al., *Local blockage of self-sustainableerythropoietin signaling supresses tumor progression in non-small lung cancer*. Oncotarget, 2017. **8**(47): p.82352-82365
124. Doleschel, D., et al., *Erythropoietin improves the accumulation and therapeutic effects of carboplatin by enhancing tumor vascularization and perfusion*. Theranostics, 2015. **5**(8): p. 905-18.

125. Scanlon, S., et al., *Multifaced Control of DNA Repair Pathwaysby the Hypoxic Tumor Microenvironment*. DNA Repair (Amst), 2016. **32**: p. 180-189
126. https://www.cancer.gov/news-events/cancer-currents-blog/2018/microbubbles-radiation-breast-cancer
127. Sharma, K., et al., *Cytotoxic Autophagy in Cancer Therapy*. Int J Mol Sci, 2014. **15**(6): p. 10034- 10051

8. Supplemental

Figure 1

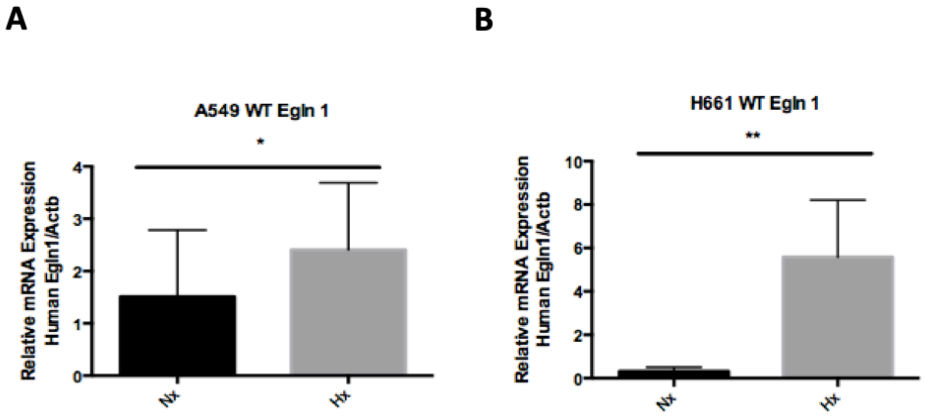


Fig. 1 Testing the hypoxic chamber.
Real Time PCR was performed using cDNA of the A549, and H661 WT in normoxic (21% O_2; black bars) and hypoxic (1% O_2; gray bars) conditions. The results obtained were normalized to β-actin (calibrator) and fold changes were calculated using the ΔΔCt method. The samples were run in duplicates. Panel **A** shows the *Egln1* expression of A549 WT cells (n=3). Panel **B** shows the *Egln1* expression of H661 WT cells (n=3). A Students t-test was performed for statistics. (Mean ± SD; n=3-4; *= 0.01≤p<0,05, **= 0.001≤p<0.01, *** = p <0.001

Figure 2

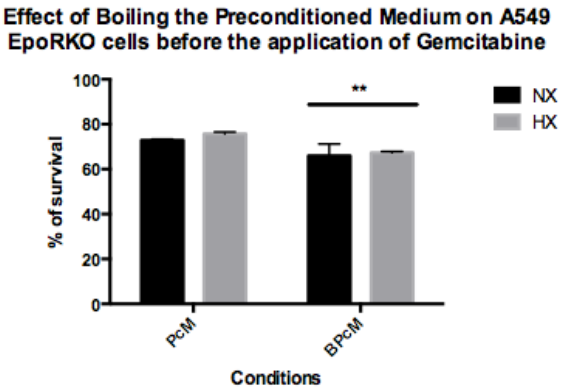


Fig. 2 Therapy resistance factor must be a protein
Figure 2 shows in normoxic (21%, black) or hypoxic (1%, gray) preconditioned medium cultivated A549 EPORKO cells (n=3) in response to Gemcitabine treatment. The preconditioned medium was either boiled before application (BPcM), or not (PcM). The survival rate was analyzed by clonogenic assay and is shown in percent (%) to untreated controls of the respective EPOR expressing or deficient cell lines. A Students t-test was performed for statistics. (Mean ± SD; n=3-4; *= 0.01≤p<0.05, **= 0.001≤p<0.01, *** = p <0.001

Figure 3

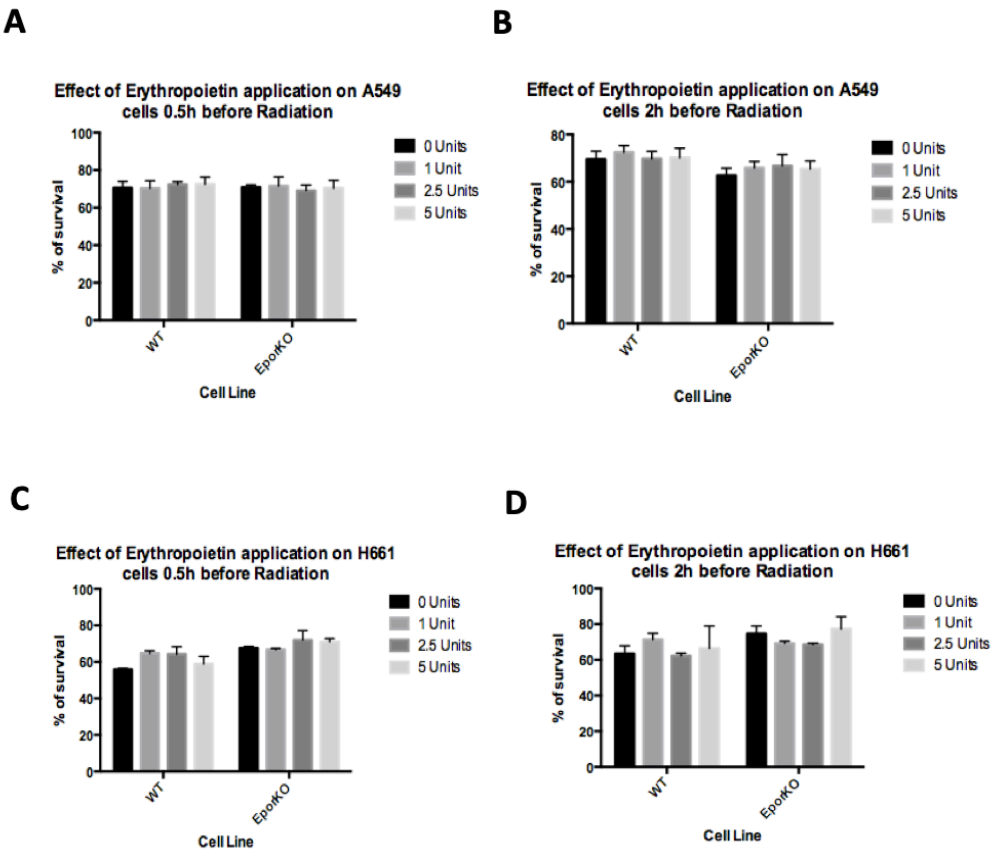


Fig. 3 Hypoxia-induced therapy resistance against Gemcitabine might be EPO independent
A549 and H661 lung cancer wildtype cells (WT) and EPO receptor knockout cells (EPORKO) were incubated either in normoxia (21% O_2, Nx) or hypoxia (1% O_2, Hx) and treated with 3 Gy radiation. The survival rate was analyzed by clonogenic assay and is shown in percent (%) to untreated controls of the respective EPOR expressing or deficient cell lines. Panels **A** and **B** show survival of in normoxia (21 %) cultivated A549 WT and EPORKO in response to 3 Gy radiation. Either 0.5h (**A**) (n=3) or 2h (**B**) (n=3) prior to radiation, cells were incubated with 1-4 u/ml EPO (gray bars) or not (black bar). Panels **C** and **D** show survival of in normoxia (21 %) cultivated H661 WT and EPORKO in response to 3 Gy radiation. Either 0.5h (**C**) (n=3) or 2h (**D**) (n=3) prior to radiation, cells were incubated with 1-4 u/ml EPO (gray bars) or not (black bar). A Students t-test was performed for statistics. (Mean ± SD; n=3-4; *= $0.01 \leq p < 0.05$, **= $0.001 \leq p < 0.01$, *** = $p < 0.001$

Figure 4

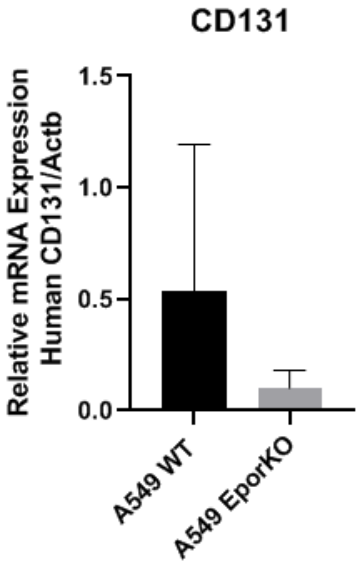


Fig 4: Expression of CD131 in A549 lung cancer cells
Real Time PCR was performed using cDNA of the A549 WT (black bar) and EPORKO (gray bar) cells to measure the expression of *CD131* of the cells. The results obtained were normalized to β-actin (calibrator) and fold changes were calculated using the ΔΔ Ct method. The samples were run in duplicates. (n=3). A Students t-test was performed for statistics. (Mean ± SD; n=3-4; *= 0.01≤p<0.05, **= 0.001≤p<0.01, *** = p <0.001

Table 4

#	Identified Proteins (76/187)	Molecular Weight	Normoxia	Hypoxia
1	Cluster of Keratin, type II cytoskeletal 1 OS=Homo sapiens OX=9606 GN=KRT1 PE=1 SV=6 (sp|P04264|K2C1_HUMAN)	66 kDa	33	32
1.1	zz|FGCZCont0077_P04264|K2C1_HUMAN blastpHomologue_0.0	TRUE		
1.2	ENSEMBL:ENSBTAP00000038253 (Bos taurus) 63 kDa protein	TRUE		
2	Cluster of Actin, cytoplasmic 1 OS=Homo sapiens OX=9606 GN=ACTB PE=1 SV=1 (sp|P60709|ACTB_HUMAN)	42 kDa	16	20
2.1	Actin, cytoplasmic 1 OS=Homo sapiens OX=9606 GN=ACTB PE=1 SV=1	42 kDa	15	19
2.2	Actin, alpha cardiac muscle 1 OS=Homo sapiens OX=9606 GN=ACTC1 PE=1 SV=1	42 kDa	10	13
3	Fibronectin OS=Homo sapiens OX=9606 GN=FN1 PE=1 SV=4	263 kDa	21	6
4	Cluster of Tropomyosin alpha-4 chain OS=Homo sapiens OX=9606 GN=TPM4 PE=1 SV=3 (sp|P67936|TPM4_HUMAN)	29 kDa	14	13
4.1	Tropomyosin alpha-4 chain OS=Homo sapiens OX=9606 GN=TPM4 PE=1 SV=3	29 kDa	13	13
4.2	Tropomyosin alpha-3 chain OS=Homo sapiens OX=9606 GN=TPM3 PE=1 SV=2	33 kDa	6	0

5	Vinculin OS=Homo sapiens OX=9606 GN=VCL PE=1 SV=4	124 kDa	11	15
6	Transforming growth factor-beta-induced protein ig-h3 OS=Homo sapiens OX=9606 GN=TGFBI PE=1 SV=1	75 kDa	16	8
7	Collagen alpha-1(I) chain OS=Homo sapiens OX=9606 GN=COL1A1 PE=1 SV=5	139 kDa	7	8
8	Clusterin OS=Homo sapiens OX=9606 GN=CLU PE=1 SV=1	52 kDa	9	8
9	Collagen alpha-1(V) chain OS=Homo sapiens OX=9606 GN=COL5A1 PE=1 SV=3	184 kDa	4	7
10	Basement membrane-specific heparan sulfate proteoglycan core protein OS=Homo sapiens OX=9606 GN=HSPG2 PE=1 SV=4	469 kDa	13	4
11	Reticulocalbin-3 OS=Homo sapiens OX=9606 GN=RCN3 PE=1 SV=1	37 kDa	5	7
12	Coactosin-like protein OS=Homo sapiens OX=9606 GN=COTL1 PE=1 SV=3	16 kDa	4	7
13	Beta-2-microglobulin OS=Homo sapiens OX=9606 GN=B2M PE=1 SV=1	14 kDa	3	5
14	Nucleobindin-1 OS=Homo sapiens OX=9606 GN=NUCB1 PE=1 SV=4	54 kDa	3	8
15	Fructose-bisphosphate aldolase A OS=Homo sapiens OX=9606 GN=ALDOA PE=1 SV=2	39 kDa	7	2
16	Collagen alpha-1(VI) chain OS=Homo sapiens OX=9606 GN=COL6A1 PE=1 SV=3	109 kDa	4	4
17	Talin-1 OS=Homo sapiens OX=9606 GN=TLN1 PE=1 SV=3	270 kDa	8	2
18	Collagen alpha-2(I) chain OS=Homo sapiens	129 kDa	4	4

	OX=9606 GN=COL1A2 PE=1 SV=7			
19	Serum albumin OS=Homo sapiens OX=9606 GN=ALB PE=1 SV=2	69 kDa	10	8
20	Plasminogen activator inhibitor 1 OS=Homo sapiens OX=9606 GN=SERPINE1 PE=1 SV=1	45 kDa	6	2
21	Galectin-3-binding protein OS=Homo sapiens OX=9606 GN=LGALS3BP PE=1 SV=1	65 kDa	2	6
22	Fatty acid-binding protein, liver OS=Homo sapiens OX=9606 GN=FABP1 PE=1 SV=1	14 kDa	2	3
23	Collagen alpha-3(VI) chain OS=Homo sapiens OX=9606 GN=COL6A3 PE=1 SV=5	344 kDa	5	2
24	Insulin-like growth factor-binding protein 2 OS=Homo sapiens OX=9606 GN=IGFBP2 PE=1 SV=2	35 kDa	3	4
25	Collagen alpha-1(XI) chain OS=Homo sapiens OX=9606 GN=COL11A1 PE=1 SV=4	181 kDa	2	3
26	Collagen alpha-1(XVIII) chain OS=Homo sapiens OX=9606 GN=COL18A1 PE=1 SV=5	178 kDa	4	3
27	Cluster of Tubulin beta chain OS=Homo sapiens OX=9606 GN=TUBB PE=1 SV=2 (sp|P07437|TBB5_HUMAN)	50 kDa	6	0
27.1	Tubulin beta chain OS=Homo sapiens OX=9606 GN=TUBB PE=1 SV=2	50 kDa	5	0
27.2	ENSEMBL:ENSBTAP00000025008 (Bos taurus) hypothetical protein	TRUE		
28	Regucalcin OS=Homo sapiens OX=9606 GN=RGN PE=1 SV=1	33 kDa	2	3
29	Phosphatidylethanolamine-binding protein 1 OS=Homo sapiens OX=9606 GN=PEBP1 PE=1	21 kDa	0	4

	SV=3			
30	Hepatocyte growth factor activator OS=Homo sapiens OX=9606 GN=HGFAC PE=1 SV=1	71 kDa	3	2
31	Cluster of Peroxiredoxin-1 OS=Homo sapiens OX=9606 GN=PRDX1 PE=1 SV=1 (sp|Q06830|PRDX1_HUMAN)	22 kDa	4	2
31.1	Peroxiredoxin-1 OS=Homo sapiens OX=9606 GN=PRDX1 PE=1 SV=1	22 kDa	2	2
31.2	Peroxiredoxin-2 OS=Homo sapiens OX=9606 GN=PRDX2 PE=1 SV=5	22 kDa	3	0
32	Alpha-actinin-4 OS=Homo sapiens OX=9606 GN=ACTN4 PE=1 SV=2	105 kDa	5	0
33	Alpha-enolase OS=Homo sapiens OX=9606 GN=ENO1 PE=1 SV=2	47 kDa	4	0
34	Golgi membrane protein 1 OS=Homo sapiens OX=9606 GN=GOLM1 PE=1 SV=1	45 kDa	0	5
35	Sulfhydryl oxidase 1 OS=Homo sapiens OX=9606 GN=QSOX1 PE=1 SV=3	83 kDa	3	2
36	Moesin OS=Homo sapiens OX=9606 GN=MSN PE=1 SV=3	68 kDa	0	4
37	Insulin-like growth factor-binding protein 4 OS=Homo sapiens OX=9606 GN=IGFBP4 PE=1 SV=2	28 kDa	0	2
38	Cluster of Tubulin alpha-1B chain OS=Homo sapiens OX=9606 GN=TUBA1B PE=1 SV=1 (sp|P68363|TBA1B_HUMAN)	50 kDa	5	0
38.1	Tubulin alpha-1B chain OS=Homo sapiens OX=9606 GN=TUBA1B PE=1 SV=1	50 kDa	4	0
38.2	Tubulin alpha-4A chain OS=Homo sapiens	50 kDa	4	0

	OX=9606 GN=TUBA4A PE=1 SV=1			
39	Laminin subunit alpha-5 OS=Homo sapiens OX=9606 GN=LAMA5 PE=1 SV=8	400 kDa	4	0
40	Histone H4 OS=Homo sapiens OX=9606 GN=HIST1H4A PE=1 SV=2	11 kDa	3	0
41	Retinol-binding protein 4 OS=Homo sapiens OX=9606 GN=RBP4 PE=1 SV=3	23 kDa	0	2
42	SPARC OS=Homo sapiens OX=9606 GN=SPARC PE=1 SV=1	35 kDa	2	0
43	Complement component C8 beta chain OS=Homo sapiens OX=9606 GN=C8B PE=1 SV=3	67 kDa	2	0
44	Peptidyl-prolyl cis-trans isomerase FKBP1A OS=Homo sapiens OX=9606 GN=FKBP1A PE=1 SV=2	12 kDa	0	2
45	Inter-alpha-trypsin inhibitor heavy chain H5 OS=Homo sapiens OX=9606 GN=ITIH5 PE=2 SV=2	105 kDa	2	2
46	Junction plakoglobin OS=Homo sapiens OX=9606 GN=JUP PE=1 SV=3	82 kDa	3	0
47	Filamin-A OS=Homo sapiens OX=9606 GN=FLNA PE=1 SV=4	281 kDa	3	0
48	14-3-3 protein zeta/delta OS=Homo sapiens OX=9606 GN=YWHAZ PE=1 SV=1	28 kDa	0	3
49	Agrin OS=Homo sapiens OX=9606 GN=AGRN PE=1 SV=6	217 kDa	3	0
50	Ferritin heavy chain OS=Homo sapiens OX=9606 GN=FTH1 PE=1 SV=2	21 kDa	2	0
51	Phosphoglycerate mutase 2 OS=Homo sapiens	29 kDa	0	2

	OX=9606 GN=PGAM2 PE=1 SV=3			
52	Tenascin-X OS=Homo sapiens OX=9606 GN=TNXB PE=1 SV=5	458 kDa	2	0
53	SH3 domain-binding glutamic acid-rich-like protein 3 OS=Homo sapiens OX=9606 GN=SH3BGRL3 PE=1 SV=1	10 kDa	0	2
54	Aldo-keto reductase family 1 member A1 OS=Homo sapiens OX=9606 GN=AKR1A1 PE=1 SV=3	37 kDa	2	0
55	Vascular cell adhesion protein 1 OS=Homo sapiens OX=9606 GN=VCAM1 PE=1 SV=1	81 kDa	0	2
56	Caldesmon OS=Homo sapiens OX=9606 GN=CALD1 PE=1 SV=3	93 kDa	0	2
57	Cathepsin Z OS=Homo sapiens OX=9606 GN=CTSZ PE=1 SV=1	34 kDa	0	2
58	Collagen alpha-1(XII) chain OS=Homo sapiens OX=9606 GN=COL12A1 PE=1 SV=2	333 kDa	2	0
59	Apolipoprotein B-100 OS=Homo sapiens OX=9606 GN=APOB PE=1 SV=2	516 kDa	3	0
60	Complement factor D OS=Homo sapiens OX=9606 GN=CFD PE=1 SV=5	27 kDa	0	2
61	Cluster of Heat shock 70 kDa protein 1A OS=Homo sapiens OX=9606 GN=HSPA1A PE=1 SV=1 (sp|P0DMV8|HS71A_HUMAN)	70 kDa	3	0
61.1	Heat shock 70 kDa protein 1A OS=Homo sapiens OX=9606 GN=HSPA1A PE=1 SV=1	70 kDa	2	0
61.2	Heat shock cognate 71 kDa protein OS=Homo sapiens OX=9606 GN=HSPA8 PE=1 SV=1	71 kDa	2	0
62	Urokinase-type plasminogen activator OS=Homo	49 kDa	2	0

	sapiens OX=9606 GN=PLAU PE=1 SV=2			
63	EGF-containing fibulin-like extracellular matrix protein 1 OS=Homo sapiens OX=9606 GN=EFEMP1 PE=1 SV=2	55 kDa	2	0
64	Glyceraldehyde-3-phosphate dehydrogenase OS=Homo sapiens OX=9606 GN=GAPDH PE=1 SV=3	36 kDa	2	0
65	Protein S100-A8 OS=Homo sapiens OX=9606 GN=S100A8 PE=1 SV=1	11 kDa	2	0
66	Putative annexin A2-like protein OS=Homo sapiens OX=9606 GN=ANXA2P2 PE=5 SV=2	39 kDa	2	0
67	Heterogeneous nuclear ribonucleoproteins A2/B1 OS=Homo sapiens OX=9606 GN=HNRNPA2B1 PE=1 SV=2	37 kDa	0	2
68	Coagulation factor XI OS=Homo sapiens OX=9606 GN=F11 PE=1 SV=1	70 kDa	2	0
69	Desmoplakin OS=Homo sapiens OX=9606 GN=DSP PE=1 SV=3	332 kDa	2	0

Table 4. Proteotomics Results
Preconditioned Medium, that was attained by preconditioning A549 WT cells in either normoxic (21% O_2) or hypoxic (1%O_2) conditions, was analyzed by the Functional Genomics Center Zürich. Highlighted yellow are the proteins that we further analyzed as alternative EPOR ligand candidates.

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Statement of authorship

I hereby declare that I have used no other sources and aids other than those indicated. All passages quoted from publication or paraphrases from these sources are indicated as such, i.e. cited and/or attributed. This thesis was not submitted in any form from another degree or diploma at any university or other institution of tertiary education.

Zürich, 27th April 2020

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